Characterisation of *Burkholderia cepacia* from clinical and environmental origins.

Thesis

Characterisation of *Burkholderia cepacia* from clinical and environmental origins.

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ABSTRACT

Burkholderia cepacia isolates were obtained from the sputum of cystic fibrosis patients or isolated from the environment. The isolates were characterised phenotypically by determining antibiotic resistance profiles and their ability to macerate onion tissue, and genetically by PCR ribotyping and macro-restriction analysis (genome fingerprinting). The replicon (chromosomal) organisation was determined by pulsed field gel electrophoresis (PFGE) and the presence of plasmids was also investigated. Plasmid transfer by conjugation was also investigated.

A high degree of genetic and phenotypic variation was found both within and between clinical and environmental populations of *B. cepacia*. Environmental isolates were generally less resistant to antibiotics but showed greater ability to macerate onion tissue. Genetic characterisation showed little evidence of acquisition of *B. cepacia* from the environment by CF patients, but revealed strong evidence supporting person-to-person transmission of *B. cepacia* in the Cardiff CF centre and other European centres.

Two or more chromosomes were found in 93 % of *B. cepacia* isolates tested, and were also found in other closely related species suggesting such organisation may be common in β-2 proteobacteria. Plasmids, often in excess of 100 kb, were found to be harboured in 52 % of isolates, though were more commonly found in CF isolates (65 % of isolates) than environmental isolates (23 % of isolates). Plasmid transfer by conjugation was demonstrated into, between and from *B. cepacia* strains. Evidence of plasmids encoding antibiotic resistance was also found

CONTENTS

PUBLICATIONS

Material contained within Chapters 3,4,5 & 8 of this thesis has been published as:

Wigley, P. & Burton, N.F. (1999) Genotypic and phenotypic relationships in *Burkholderia cepacia* isolated from cystic fibrosis patients and the environment. *Journal of Applied Microbiology* in press.

Material contained in Chapter 9 forms part of a paper submitted for publication:

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LIST OF ABBREVIATIONS

NA	nutrient agar
NB	nutrient broth
DST	diagnostic sensitivity test
LB	Luria-Bertani broth
EDTA	ethylenediaminetetra acetic acid
Tris	tris(hydroxylmethyl) methylamine
SDS	sodium dodecyl sulphate
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
MLEE	multilocus enzyme electrophoresis
RFLP	restriction fragment length polymorphism
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
NCIMB	National Collection of Industrial and Marine Bacteria
NCPPB	National Collection of Plant Pathogenic Bacteria
NCTC	National Collection of Type Cultures
ATCC	American Type Culture Collection
v/v	Volume/volume
w/v	weight/volume
MIC	minimum inhibitory concentration
$V\ cm^{-1}$	Volts per centimetre
bp	base pairs
kb	kilobase pairs
Mb	megabase pairs
Da	Daltons
kDa	kilodaltons
MDa	megadaltons

Chapter 1 General introduction

1.1 *Burkholderia cepacia*

Burkholderia cepacia is a Gram negative rod-shaped bacterium. It is important clinically, particularly causing respiratory infections in cystic fibrosis patients, and in the environment as a phytopathogen and as a potential agent of bioremediation and biocontrol (Govan *et al* 1996).

1.1.1 History and taxonomy

In 1950 Walter H Burkholder first described the causative organism of "Sour skin" onion rot, commonly found in New York state, U.S.A. This is a bacterial rot that affects onion bulb scales resulting in a characteristic sour, vinegar-like smell, hence its name. He described the pathogen as a yellow pigmented, non-fluorescent, Gram negative, non-spore-forming rod of around 1to2.8 μm in length, with 1 to 3 polar flagellae. His description placed the bacterium within the genus *Pseudomonas* and so he named it *Pseudomonas cepacia* from the Latin noun *caepa* or *cepa* meaning onion. Burkholder had previously described 2 other phytopathogenic pseudomonads. *Ps. allicola* and *Ps. carophylli*, now known to be close relatives of *B. cepacia*.

In 1959 Morris and Roberts published studies on unnamed organisms isolated from water and soils in Trinidad. Stanier and co-workers (Stanier *et al* 1966) further studied the strains of Morris and Roberts along with clinical and further soil isolates. They named the organism *Ps. multivorans* (Latin-eater of everything), based upon the ability of the strains to utilise a large range of organic compounds as sole carbon and energy

sources. In 1970 synonymy between *Ps. multivorans* and *Ps. cepacia* was established (Ballard *et al* 1970), and its description enlarged upon. In the same year Jonsson proposed a new species *'Ps. kingii'* based on the work of King who had previously described an organism found in clinical isolates as Eugonic oxidiser (EO -1) as it could grow on most media and use most carbohydrate sources (King 1964, Jonsson 1970). In 1972 *"Ps. kingii"* was established (Snell *et al* 1972) as a synonym of *Ps. cepacia*, giving *Ps. cepacia* priority in the nomenclature.

In 1973 Palleroni and co-workers divided the genus *Pseudomonas* into five groups, as there were great differences of phenotypic properties within the genus. The categorisation was based upon rRNA-DNA hybridisation, with the genus type species *Ps. aeruginosa* placed into RNA homology group I. *Ps. cepacia* was placed into group II along with four other phytopathogens. *Ps. gladioli* (previously known as *Ps. marginata* or *Ps. allicola*), *Ps. carophylli*, *Ps. solanacearum* and *Ps. pickettii*, and the animal pathogens *Ps. mallei* and *Ps. pseudomallei.*

The name *Ps. cepacia* was omitted from the Approved List of Bacterial names in 1980 and so lost its validity, however a year later Palleroni and Holmes (1981) revived the name on the basis of Ballard's work a decade earlier. Grouping by the methods of Woese (1987) placed this group in the β-proteobacteria with species such as *Alcaligenes eutrophus*, and *Ps. aeruginosa* in the γ-proteobacteria group.

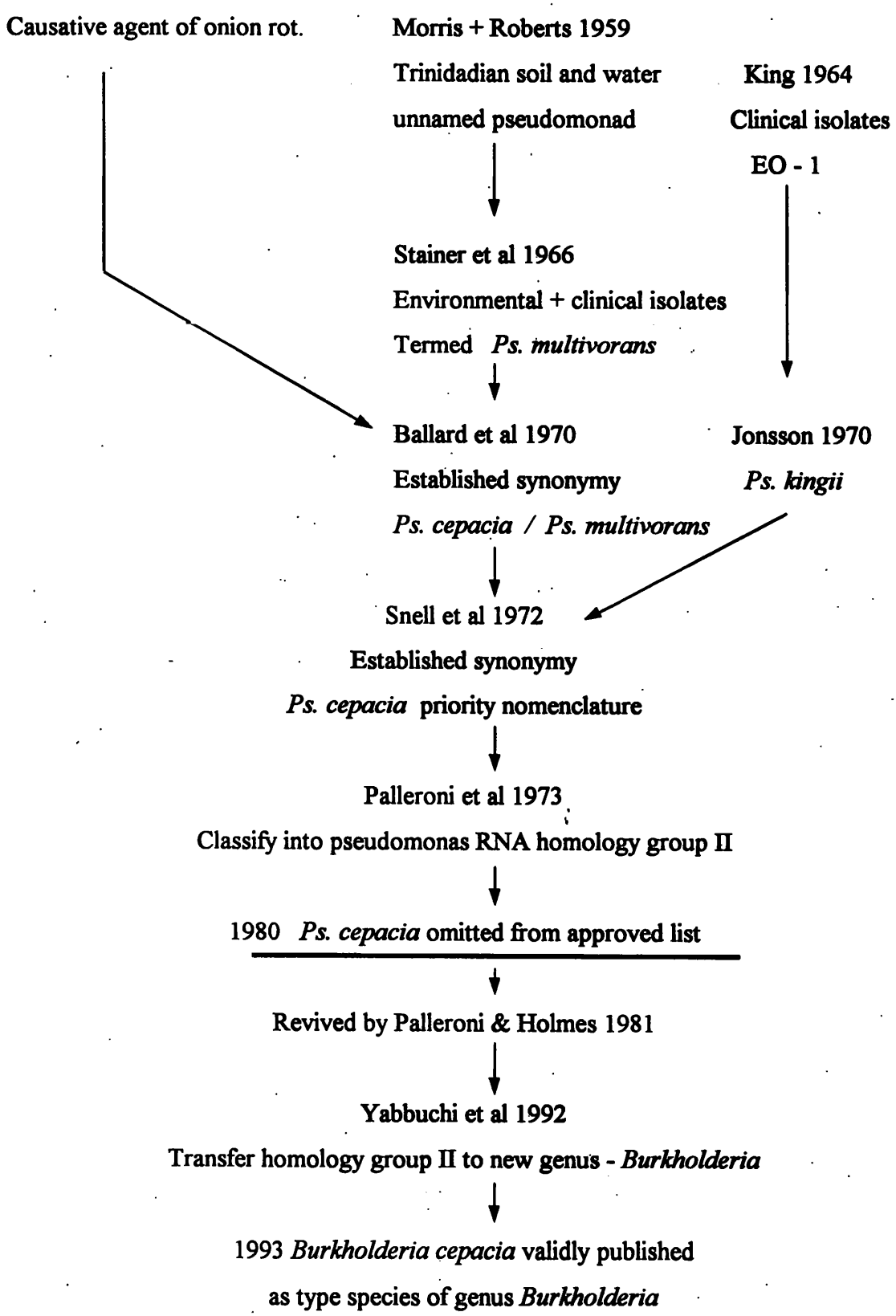
In 1992 it was proposed that the RNA homology group II should be transferred to the new genus *Burkholderia*, after W. H. Burkholder (Yabuuchi *et al* 1992). The transfer

was based upon similarities in the seven constituent members' 16S rRNA sequences, fatty acid and cellular lipid composition and phenotypic characteristics, as compared with other members of the *Pseudomonas* genus, in particular the type species *Ps. aeruginosa*. Therefore seven new combinations: *B. cepacia, B. gladioli, B. carophylli, B. solanacearum, Burkholderia pickettii, B. mallei* and *B. pseudomallei* were proposed. In 1993 these were validated (List No 45 Int. J. Syst. Bacteriol) with *B. cepacia* as the type species. Subsequently several other *Pseudomonas* and newly described species have been placed in the genus *Burkholderia* including *B. plantarii, B. glumae, B. vandii* and *B. vietnamensis* (Urakami *et al* 1994).

Recent work has highlighted great phenotypic and genetic variation within the *B. cepacia* nomenspecies. A study of metabolic properties in a range of clinical and environmental isolates of *B. cepacia*, including catabolic properties and phytopathogenicity to onion, gave four phenetic clusters or phenoms upon statistical analysis of the results (Yohalem and Lorbeer 1994). DNA-DNA and DNA-ribosomal RNA (r-RNA) hybridisation analysis, along with analysis of fatty acid and cellular protein composition revealed that *B. cepacia* isolates from cystic fibrosis patients in Belgium and the UK fell in three distinct groups, different from the type strain of the species (Vandamme 1995, Revets *et al* 1996, Govan *et al* 1996). Differing genomic groups within a nomenspecies that cannot be readily differentiated phenotypically at present have been proposed to be called genomovars (Ursing *et al* 1995). It was proposed that genomovars should be numbered with the species type strain being placed in genomovar I. It is also suggested that each genomovar should have an individual type strain. In *B. cepacia* the species type strain ATCC 25416 (NCPPB

2993) has been placed into genomovar I together with the majority of environmental isolates so far analysed by DNA-DNA hybridisation (Govan *et al* 1996). The majority of CF isolates group into genomovar III. Subsequently it has been proposed that *B. cepacia* be divided into five distinct genomic groups, with genomovar II being re-classified as a separate species, *Burkholderia multivorans,* within the *B. cepacia* complex (Vandamme *et al* 1997). It has also been proposed that several of the genus *Burkholderia* be re-assigned to the newly proposed genus *Ralstonia* (Yabuuchi *et al* 1995), and that *Ps. phenazium, Ps. pyrrocinia* and *Ps. glathei* be transferred to the genus *Burkholderia* along with the newly discovered rhizospheric bacterium *Burkholderia graminis* (Vandamme *et al* 1997; Viallard *et al* 1998).

Figure 1.1 History and Taxonomy of *Burkholderia cepacia*.



1.1.2 Characteristics of B. *cepacia*

The characteristics of *B. cepacia* are much as originally described by Burkholder (Palleroni, 1984). It is an aerobic, glucose non-fermenting rod, 1.6 to 3.2 μm long, 0.8 to 1.0 μm in diameter. It is non-fluorescent and may produce a green-yellow pigment, though more frequently colonies are white or cream in colour.

B. cepacia is an ubiquitous organism. It has been found in water and soils (Morris and Roberts 1959), plants (Burkholder 1950), a wide range of fomites in hospitals and a number of clinical specimens (Gilardi 1983, Holmes 1986).

B. cepacia is biochemically diverse and can oxidise a large number of compounds as a carbon source. A large number of carbohydrates and polyalcohols may be utilised as well as a diverse range of compounds including Penicillin G, aromatic compounds, and chlorinated aromatic compounds recalcitrant to biodegradation (Holmes 1986).

Plasmids appear to play a role in some of these catabolic processes. A role has been demonstrated in the degradation of the herbicide 2,4 dichlorophenoxyacetate (2,4 -D) (Bhat *et al* 1994) and degradation of toluene (Shields *et al* 1995) in strains of *B. cepacia.*

B. cepacia is resistant to several disinfectants including solutions of benzalkonium chloride and chlorhexidine gluconate (sold under the tradename of Savlon) (Holmes 1985). Resistance to a wide range of antimicrobial agents is also found (discussed in detail later).

1.1.3 Genetic organisation of *B. cepacia*

Relatively little is known about genetic organisation in *B. cepacia*. It is apparent that the genome contains large numbers of insertion sequences and that these play a role in the genomic plasticity of *B. cepacia* and its' degradative ability (Byrne and Lessie 1994, Cheng and Lessie 1994). However there is little known regarding the distribution of these insertion sequences or gene organisation.

Genome organisation

B. cepacia has a large genome of around 6.5 Mb to 8.1 Mb. This is larger than the 5.8 Mb genome of *Ps aeruginosa* and is around twice the size of the average prokaryotic genome (Grouthes and Tummler 1991, Cheng and Lessie 1994, Rodley *et al* 1995). The use of pulsed field gel electrophoresis has revealed that the genome of *B. cepacia* is arranged into multiple circular replicons rather than a single circular chromosome (Cheng and Lessie 1994, Rodley *et al* 1995). Cheng and Lessie (1994) described the organisation of the genome of *B. cepacia* strain ATCC 17616 into three circular replicons of 3.4, 2.5 and 0.9 Mb, along with a cryptic plasmid of 170 kb, giving an overall genome size of 7.0 Mb. Southern hybridisation studies showed that each of the large replicons contained rRNA genes and previously identified insertion sequences. Rare cutting restriction enzymes were used to map the replicons. The transposon Tn*5-751s* containing a *Swa* I recognition site was used to inactivate and hence map the position of genes by insertional mutagenesis. This indicated that the 3.4 Mb replicon contains genes encoding for the amino acids isoleucine, arginine, histidine and threonine, whilst the 2.4 Mb replicon contains genes encoding for the synthesis of lysine, β-lactamase activity and the use of ribital and trehalose as carbon sources.

Rodley and co-workers (1995) produced a restriction map of the *B. cepacia* type strain ATCC 25416 (or NCPPB 2993). They also described three large circular replicons of 3.65 Mb, 3.17 Mb and 1.07 Mb along with a 200 kb plasmid, a total genome size of 8.1 Mb. Three other *B. cepacia* strains were also analysed. These gave multiple replicons of 3.5 Mb and 1.0 Mb, though analysis of restriction fragments showed the genome to be somewhat larger suggesting that the 3.5 Mb replicon band may be two replicons of similar size. Other closely related bacteria of the β-2 subdivision of proteobacteria (Woese 1987) were also demonstrated to have multiple replicons. *B. glumae* contained replicons of 3 and 3.8 Mb, whilst *B. picketti, B. solanacereum, Alcaligenes eutrophus* and *Ps. glathei* all contained more than one replicon, though nothing was detected in *B. gladioli*. Strains from the neighbouring and more distantly related β-1-subdivision did not appear to contain multiple replicons. Lessie *et al* (1996) have subsequently determined the replicon organisation of 12 *B. cepacia* strains. Between two and four replicons were found with a total genome size of 4.6 to 8.1 Mb.

The presence of multiple replicons differs from the traditional view of a single circular chromosome in bacteria though there is evidence for multiple replicons in the afore mentioned species and in unrelated species including α proteobacteria such *Rhizobium meliloti* where three circular replicons of 3.4, 1.7 and 1.4 Mb have been observed (Sorbal *et al* 1991,Honeycutt *et al* 1993), *Agrobacterium tumefaciens* which appears to have both a circular 3 Mb and linear 2.1 Mb chromosome (Allardet-Servet *et al* 1993) whilst linear chromosomes have been described in *Borrelia* and *Streptomyces* (Hinnebusch and Tilly 1993).

Insertion sequences

B. cepacia has a genome rich in insertion sequences or IS elements (Cheng and Lessie 1994). IS elements are small (less than 2 kb) transposable elements that carry no genes unrelated to their insertion function (Reviewed by Iidia *et al* 1983). IS elements have been identified in many prokaryotic and eukaryotic organisms. Their ability to undergo transposition (homologous recombination-independent transfer) to new sites and replicons and duplication are characteristic features that have genetic consequences in deletion, insertional inactivation, fusion of independent replicons and transcriptional activation of genes (Haugland *et al* 1990).

The IS elements in *B. cepacia* has implications in the promotion of genetic rearrangement of plasmid and chromosomal replicons (Byrne and Lessie 1994). IS elements have been found to play a role in gene activation by increasing expression of the β-lactamase gene on the plasmid pRP1 up to thirty fold (Scordilis *et al* 1987), in activation of the transposon Tn*1* *bla* gene on plasmid pTGL52 (Ferrante and Lessie 1991) and in the activation of *lac* genes in *B. cepacia* and other Gram-negative bacteria (Wood *et al* 1990, Wood *et al* 1991). IS elements are thought to play a role in obtaining foreign genes for catabolic functions such as degradation of 2,4,5 trichloro-phenoxyacetic acid (2,4,5,-T) (Haugland *et al* 1990) or β-lactam antibiotics (Scordilis *et al* 1987).

The above evidence has given rise to the thought that IS elements play a role in the broad catabolic and biodegradative properties of *B. cepacia* as well as its genomic plasticity both by genetic rearrangement and recruitment of foreign DNA from

conjugative plasmids and transposons to develop degradative pathways, and by the ability to express 'foreign' genes at high levels (Cheng and Lessie 1994).

1.1.4 Clinical Importance of *B. cepacia*

In the 1980's *B. cepacia* emerged as an important pathogen in CF respiratory disease. Reports from Canada (Isles *et al* 1984) described an adverse outcome for a number of patients. Such reports were confirmed in centres in N. America (Tablan *et al* 1985, Thomassen *et al* 1985) and in the UK (Simmonds *et al* 1990).

An increase in affected patient numbers throughout the late 1980's into the early 1990's have given rise to great concern, particularly in the light of growing evidence of person to person transmission. (Millar-Jones *et al* 1992, Govan *et al* 1993, Walters and Smith 1993). Of particular concern is evidence of strains causing a necrotising pneumonia (Tomashefski *et al* 1988) and the high levels of antimicrobial resistance displayed by the organism (Santos Ferreira *et al* 1985, Kumar *et al* 1989).

Although *B. cepacia* infection is an increasingly serious problem in CF, it is generally regarded as a low grade pathogen. *B. cepacia* is not a frequent human pathogen, most isolates being present as saprophytes or commensals. However *B .cepacia* may act as an opportunistic agent in compromised patients such as diabetics, intravenous drug users, alcoholics and immunocompromised patients as well as cystic fibrosis sufferers. (Gilardi 1983). The ability of *B. cepacia* to grow in antiseptic, anaesthetic and disinfectant solutions, as well as distilled or deionized water, mark it down as a potentially dangerous source of nosocomial infection.

In 1986 Holmes reviewed the sources of 195 strains of *B. cepacia* sent to the national collection of type cultures (NCTC) for identification. These represented around 4% of the total Gram negative, non fermenting bacteria sent for identification. Most clinical isolates were isolated in the US or UK from sources including blood, sputum, vaginal fluid, urine, bronchial washings and synovial fluid. The majority of clinical isolates were obtained from compromised patients, though not CF patients, whilst a number were isolated from contaminated medical materials such as disinfectants. Amongst the cases reviewed urinary tract infections had occurred from the use of contaminated catheters or catheter irrigation fluid, though these were largely asymptomatic and most ceased upon removal of the catheter, septicaemia with endocarditis caused by *B. cepacia* had arisen following heart surgery. *B. cepacia* endocarditis, sometimes fatal, has also been reported in intravenous drug users. *B. cepacia* septicaemia has also been fatal in burns patients and wound infections have occurred due to the application of contaminated antiseptic and disinfectant solutions, whilst a case of septic arthritis was reported following the intra-articular injection of contaminated methyl-prednisilone. More recently an outbreak of *B. cepacia* bacteremia occurred in an oncology clinic in Alabama (Pegues *et al* 1993). This case was traced back to a contaminated heparin flush solution, used to flush venous catheters.

Several cases of respiratory disease in non-CF patients have been reported (Holmes 1986) though in each case the patient was found to be compromised in some way. An individual developed a case of necrotising pneumonia after cleaning air conditioning systems. After treatment he recovered but the pneumonia recurred. Upon further investigation a phagocyte dysfunction was found. In children with a similar condition,

chronic granulomatous disease (CGD), there is an association with *B. cepacia* colonisation and pneumonia in several reported cases(Speert *et al*1994). A fatal pneumonia has also been described in a diabetic, after therapy with an ultrasonic nebuliser. The source of *B. cepacia* in this case was found to be contamination of the nebuliser reservoir.

1.2 Cystic fibrosis and microbiology of CF

1.2.1 Cystic fibrosis

Cystic fibrosis is the most common lethal hereditary disease in caucasian populations. It is typified by thick secretions and recurrent bacterial respiratory infections. The disease is the result of an abnormal chloride channel protein. Anderson (1938) first described Cystic Fibrosis (CF) in a group of 49 children who failed to thrive despite feeding. The children had "distended abdomens and attacks of diarrhea with large, pale, foul smelling stools". The stools were found to have a high ratio of "split fats". Children who died in the neonatal period were found to have lesions of the pancreas, hence the term "Cystic Fibrosis of the Pancreas" was coined.

CF most commonly affects people of European origins, affecting around 1 in 2000 live births in caucasians. (Tsui 1992) The gene carrier frequency is estimated to be between 2 to 5 % of the general caucasian population (Tsui 1992). It is far less common in Blacks and Orientals where the incidence is about 1 in 100,000 live births. The suggestion has been put forward that the prevalence in the caucasian population may be due to a selective advantage for CF gene carriers (Tsui 1992).

CF occurs as a result of abnormal secretion. This is due to a chloride channel defect in epithelial cells The secretions contain too little water relative to the proteins and electrolytes within them. This results in the characteristic thick mucus (mucoviscoidoses), and also the high concentrations of sodium and chloride in sweat. The measurement of sodium in sweat is the main diagnostic test for CF.

The clinical manifestations of CF can range from a mild, almost asymptomatic condition, to a very severe disease causing death within a year of birth. The most serious problem is respiratory tract infection. Because of the inability of the patient to clear the thick, viscous mucus in the bronchial airway, the patient is susceptible to recurrent infections by a range of micro-organisms, including *Staphylococcus aureus, Ps. aeruginosa, Haemophilus influenzae* and *B. cepacia*. The establishment of encapsulated, antibiotic resistant strains of bacteria cause a progressive deterioration of lung tissue and eventually to loss of lung function; the most common cause of death.

In children the disease commonly presents with recurrent upper and lower respiratory tract infection resulting in bronchiectasis, the persistent dilation of bronchi and bronchioles (Wormsley1986). This is usually saccular in nature, and is typified by coughing with purulent sputum, wheezing, progressive dyspnoea and finger clubbing. In adults bronchiectatic infection is also common. This may be accompanied by pneumothorax, respiratory failure and corpulmonale.

Pancreatic insufficiency is the second most important clinical manifestation of CF. In many cases from early in life, or even at birth, the pancreatic ducts may become blocked by impissated secretions. This leads to dilation of the ducts, followed by inflammation, and then to deterioration of the pancreas leading to the loss of the digestive enzyme producing acinar tissue, which is replaced by fat and fibrous tissue. The result is pancreatic exocrine insufficiency, which may lead to steatorrhoea, creatorrhoea and diarrhoea. The reduction in production of pancreatic juice containing amylase, protease and lipase digestive enzymes, along with possible blockage of the bile

duct leads to digestive disorders, such as those above, and may lead to undernutrition in infants. It also explains the "high level of split fats" in stools, described by Anderson (1938)

The genetic defect leading to CF was mapped to a single gene on chromosome 7 band q31. The function and predicted structure of the CF gene product has been reviewed extensively by Riordan (1993). The resulting product is a cAMP induced chloride channel expressed in epithelial cells known as the Cystic Fibrosis Transmembrane Regulator (CFTR). By late 1992 more than 230 different CFTR mutations had been found (reviewed by Tsui 1992). Initially it had been thought that only a few mutations might occur, as suggested by the ΔF508 mutation, a mutation in a phenylalanine residue at position 508, occurring in around 70% of CF patients and the strong association found between haplotype and allele. The majority of CF patients have a form of the disease as caused by the ΔF508 mutation. At the end of 1992 some 30,000 mutant chromosomes had been analysed world-wide (Tsui 1992). 67% were found to carry the ΔF508 mutation, with only another 7 mutations occurring in more than 100 cases. The frequency and distribution of CFTR mutations also seems to follow a s pattern. In parts of Scandinavia and the UK the ΔF508 mutation runs in over 90% of CF patients. This falls in a south easterly direction to 55% in Milan and as low as 22% in the Ashkenazic Jewish population of Jerusalem. In the latter group, 60% of CFTR mutations are the W1282X mutation, found in 2% of the world-wide CF population (Tsui 1992).

The use of gene therapy in CF has been proposed, with both adenovirus and liposome delivery systems having been used in limited trials (Johnson 1995). However this has been of limited success, with no immediate prospect of clinical use.

1.2.2 Microbiology of C.F.: general considerations

Chronic progressive lung disease due to bronchopulmonary infection is the most important pathological consequence of CF; 90 % of deaths result from bronchopulmonary disease. As a result of the defective CFTR gene, there is viscous mucus found in the airways and along with defects in respiratory epithelia this leads to defective mucocilliary clearance of infective agents, and hence the pathological consequences discussed earlier. The microbiology of these infective agents has been reviewed by Gilligan (1991) and Govan and Nelson (1992). It has been recently proposed that the defective CFTR -associated chloride transport may lead to colonisation of bacteria in the lung. (Smith *et al* 1996). Normal lung epithelium appears to have a bactericidal effect against both *S. aureus* and *Ps. aeruginosa* but the high sodium chloride concentrations found in CF inhibit this, leading to decreased ability to kill the bacteria and increased susceptibility to infection.

Upon birth, the lungs of CF patients are histologically normal, but infections are soon detected. These occur mainly in the major and minor airways rather than the alveoli. The infections are insidious and recurrent, eventually leading to chronic colonization with bacteria. A relatively narrow spectrum of infectious agents infect CF patients, however as antimicrobial therapy has led to an increased life expectancy, the spectrum

of opportunistic and infective agents has increased, most notably to now include *B. cepacia* and *Mycobacterium.*

The bacteria most commonly found to cause chronic infection in CF are *S. aureus* and *Ps. aeruginosa.* In a 1986 survey of US CF Centres, 60 % of patient's sputum cultures showed *Ps. aeruginosa* and 27% contained *S. aureus. Haemophilus influenzae* is found in around 10-15% of cases, whilst *B. cepacia* is an increasing problem. In addition non-glucose fermenters (*Legionella pneumophilia* and *Mycobacterium spp*) viruses and fungi, especially *Aspergillus spp.* have been implicated in cases of CF lung disease.

Generally patterns of infection follow a sequence throughout the patients life. This begins with *S. aureus* colonization in infancy, frequently followed by *H. influenzae.* In early adolescence *Ps. aeruginosa* infection becomes most common, whilst *B. cepacia* and mycobacteria have their highest incidence in young adults.

1.2.3 *Staphylococcus aureus*

S. aureus was the first organism described as causing chronic lung disease in CF, (Anderson 1938) and was the biggest cause of death in CF patients prior to the development of antimicrobial chemotherapy. Few CF patients survived beyond infancy due to *S. aureus* infection, and it is still of great importance in CF patients under the age of 10. The virulence of *S. aureus* in CF depends on 2 main factors; the ability to adhere to respiratory epithelium and the ability to evade immune clearance though the exact mechanisms of adherence are not known (Govan and Nelson 1992). Anti-

staphylococcal therapy has proved effective in CF. β-lactam drugs such as oxacillin and dicloxacillin, fusidic acid, co-trimoxazole, tetracyclines and first generation cephalosporins have all proved effective. (Gilligan 1991, Govan and Nelson 1992). The widespread and intense use of antimicrobial therapy in CF *S. aureus* infection is somewhat controversial, particularly where it is used prophylactically. In Edinburgh treatment for *S. aureus* is only used when a high sputum carriage (>107 cfu ml^{-1}) is found (Govan & Nelson 1992). The use of prophylaxis may lead to the development of resistant strains. This seems particularly to be the case with the use of co-trimoxazole (trimethoprim-sulphamethoxazole) which can lead to thymidine dependent resistant strains. It has also been suggested that antimicrobial therapy acts 'to prime' the lungs for subsequent *Ps. aeruginosa* and *Haemophilus influenzae* colonization.

1.2.4 *Pseudomonas aeruginosa*

With the development of effective anti-staphylococcal therapy, *Ps. aeruginosa* has become the most important infection in CF. Infection by *Ps. aeruginosa* in CF has two key features. The first is that colonization remains restricted to the lungs and the second is phenotypic adaptation occurs within the lung. The most evident phenotypic characteristic is the appearance of mucoid variants formed by the production of large amount of exopolysaccharide alginate. This alginate appears to play a role in cellular adhesion, protection against immune clearance and indirect tissue damage (Gilligan 1991, Govan and Nelson 1992).

Colonisation of CF airways by *Ps. aeruginosa* seems usually to be by non-mucoid forms. After a period of time, sometimes 3 months or less, mucoid variants are found

to occur. Chronic colonization generally involves a single strain of *Ps. aeruginosa* that phenotypically varies over a period of time, though in some cases more than one strain may be carried. It is thought that the upper respiratory tract is initially colonised, leading to the eventual colonization of the lower airways. This is supported by evidence of *in vitro* binding and in patients who, following heart-lung transplantation, have a recurrent infection with the same strain of *Ps. aeruginosa* that colonised pre-transplant. This suggests an upper tract reservoir of the bacterium (Gilligan 1991, Govan and Nelson 1992).

The persistence of *Ps. aeruginosa* in CF lung infection appears to be due to the high amounts of alginate produced by mucoid variants. The production of the 'mucoid exopolysaccharide, or MEP, seems to be governed by a chromosomal region designated *muc* or *alg ST.* The conversion of non-mucoid to mucoid phenotype seemingly occurs as a result of a mutation in the *muc* region, in response to environmental stimuli in the lung. The alginate produced is an anionic polysaccharide formed of 1-4 linked β-D-mannuronic acid and α-L-guluronic acid. It is highly viscous, binding to water and divalent cations to form a gel. This appears to happen in CF, where post-mortem lung material has revealed *Ps. aeruginosa* microcolonies enclosed in a larger alginate matrix. This can be explained by gel formation due to interaction with the high levels of calcium ions found in CF lungs. The resulting 'gel enclosed' microcolonies are even more difficult to remove by mucocilliary clearance, and so give rise to persistent *Ps. aeruginosa* colonization. In addition, increased damage to the lungs occurs due to 'frustrated phagocytosis'. Immune complex formation and presence of immunoglobulins stimulates phagocytes, in particular neutrophils, which when

staphylococcal therapy has proved effective in CF. β-lactam drugs such as oxacillin and dicloxacillin, fusidic acid, co-trimoxazole, tetracyclines and first generation cephalosporins have all proved effective. (Gilligan 1991, Govan and Nelson 1992). The widespread and intense use of antimicrobial therapy in CF *S. aureus* infection is somewhat controversial, particularly where it is used prophylactically. In Edinburgh treatment for *S. aureus* is only used when a high sputum carriage (>107 cfu ml^{-1}) is found (Govan & Nelson 1992). The use of prophylaxis may lead to the development of resistant strains. This seems particularly to be the case with the use of co-trimoxazole (trimethoprim-sulphamethoxazole) which can lead to thymidine dependent resistant strains. It has also been suggested that antimicrobial therapy acts 'to prime' the lungs for subsequent *Ps. aeruginosa* and *Haemophilus influenzae* colonization.

1.2.4 *Pseudomonas aeruginosa*

With the development of effective anti-staphylococcal therapy, *Ps. aeruginosa* has become the most important infection in CF. Infection by *Ps. aeruginosa* in CF has two key features. The first is that colonization remains restricted to the lungs and the second is phenotypic adaptation occurs within the lung. The most evident phenotypic characteristic is the appearance of mucoid variants formed by the production of large amount of exopolysaccharide alginate. This alginate appears to play a role in cellular adhesion, protection against immune clearance and indirect tissue damage (Gilligan 1991, Govan and Nelson 1992).

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to occur. Chronic colonization generally involves a single strain of *Ps. aeruginosa* that phenotypically varies over a period of time, though in some cases more than one strain may be carried. It is thought that the upper respiratory tract is initially colonised, leading to the eventual colonization of the lower airways. This is supported by evidence of *in vitro* binding and in patients who, following heart-lung transplantation, have a recurrent infection with the same strain of *Ps. aeruginosa* that colonised pre-transplant. This suggests an upper tract reservoir of the bacterium (Gilligan 1991, Govan and Nelson 1992).

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confronted by the relatively huge microcolonies release reactive oxygen radicals or elastases and so cause immune mediated damage to the lungs. The wide range of virulence factors produced by *Pseudomonas aeruginosa* also contribute to the pathology seen in CF lungs (Gilligan 1991, Govan and Nelson 1992).

1.2.5. *Haemophilus Influenzae*

Respiratory disease due to non-capsulated, non-typable *H. influenzae* is often undiagnosed. Surveys have found a frequency of 10-14% in CF sputum, and it is found particularly in young children. It is often undiagnosed due to the difficulty of culture and difficulty in distinguishing from commensal *H. influenzae.* Although a contributory factor to CF lung disease *H. influenz*ae does not persist within the lung, and does not seem to play a primary role in CF respiratory disease (Gilligan 1991, Govan and Nelson 1992).

1.2.6. *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia (formerly *Xanthomonas maltophilia*) appears to be an emerging problem amongst CF patients with prevalence rate of up to 30% in some CF centres (Denton 1997). Little is known about epidemiology or virulence factors in *Sten. maltophilia* infections, though like *B. cepacia* high levels of antibiotic resistance. The mechanisms of disease appear to be similar to those in *Ps. aeruginosa*. *Sten. maltophilia* has been mis-identified as *B. cepacia* (Burdge *et al* 1995).

1.2.7 Other bacterial, fungal and viral agents

Although the role of mycobacteria in CF has not been studied to any great extent, fatal disease in CF patients has been reported as a result of opportunistic infection by the atypical mycobacteria *M. fortuitum* and *M. chelonei.* Infection with *M. tuberculosis* is very rare in CF. Patients should be monitored for possible mycobacterial infections. *Streptococcus pneumoniae* and enteric bacteria have all been cultured from CF respiratory secretions. Their clinical significance, if any, is not known.

Aspergillus fumigatus is the most important fungal agent associated with CF. It is estimated that up to 80% of CF patients may be colonised with *A. fumigatus.* Between 0.5 to 11% of patients develop non-invasive allergic bronchopulmonary aspergillosis (ABPA). Around a third of CF patients have precipitating antibodies to *A. fumigatus.* Invasive infections are rare. Treatment for ABPA is usually by corticosteroids (Dunnill 1982). *Candida albicans* is often found in CF respiratory tracts, particularly in patients undergoing antimicrobial or steroid therapy, though its' role in disease is not defined.

The role of viruses in CF is not yet known. Respiratory syncytial virus (RSV) and influenzae infections are common, and it has been suggested they play a 'priming' role for subsequent bacterial infection (Gilligan 1991, Govan and Nelson 1992).

1.3 *B. cepacia* in cystic fibrosis

B. cepacia had been cultured from the sputum of CF patients throughout the 1970's but was regarded as little more than a harmless commensal of little clinical significance. However in 1984 a report from Toronto, Canada (Isles *et al* 1984) outlined the emergence of *B. cepacia* as an increasing problem in CF patients. At this CF centre the carriage rate of *B. cepacia* increased from 10% in 1971 to 18% in 1981. *B. cepacia* was also found to cause a more severe pulmonary disease than *Ps. aeruginosa*, further compounded by difficulty in treatment due to the high levels of antimicrobial resistance.

A similar pattern of *B. cepacia* infection was also found in Cleveland U.S.A. (Thomassen *et al* 1985), where an increase in prevalence in CF patients from 7% in 1978 to 15% in 1983 occurred. Of considerable cause for concern was the increasing number of patient deaths associated with *B. cepacia*. In 1978 9% of deaths were associated with *B. cepacia*, five years later this had risen to 55%. Over the 5 years of the study 85 chronic and 31 non-chronic cases of *B. cepacia* infection were observed at this CF centre. The patients were classified by chronic/non-chronic status, and by the clinical course of the infection. The patients were classified into 3 groups:-

Group I - Patients chronically colonised with *B. cepacia* who did not undergo change in clinical condition. These patients were in good, moderate or poor condition at the time of colonisation.

Group II - Patients chronically colonised with *B. cepacia* who continued to decline clinically. These patients were in moderate or poor condition at the time of colonisation.

Group III - Patients chronically colonised with *B. cepacia* who underwent a rapid and unexpected clinical decline ending in death. These patients were in good or moderate condition at time of colonisation.

The increase in *B. cepacia* infection and mortality was also observed at the Philadelphia CF centre (Tablan *et al* 1985).

In the United Kingdom an increase of *B. cepacia* infection to a maximum prevalence of *B. cepacia* infecting 7%of patients was observed at the Leeds CF centre (Simmonds *et al* 1990). 27% of *B. cepacia* infected patients died, compared with 7% chronically infected by *Ps. aeruginosa*. Isolation from CF patients of *B. cepacia* in both the UK and N. America CF centres have risen in the early part of the nineties, some CF centres reporting a prevalence of up to 40% in CF patients (Anonymous 1992, Tablan 1993) though overall prevalence rates in the U.S.A. seem to have levelled out at 3.5%, with an annual incidence of new cases rate of 1% amongst CF patients.

1.3.1 *B. cepacia* respiratory disease

The role of *B. cepacia* in CF lung disease has proved somewhat controversial. In some quarters it has been suggested that *B. cepacia* is merely a commensal, and acts as a marker for underlying disease (Gilligan 1991). However the current weight of opinion is that *B. cepacia* can cause either a non-chronic transient infection, or a chronic infection following one of three clinical pathways initially described by Thomassen and co-workers (1985, Govan & Nelson 1992)

The three clinical courses of chronic *B. cepacia* infection are

1. Chronic asymptomatic carriage

2. Progressive deterioration

3. Rapidly fatal deterioration

The first two of these categories resemble other CF respiratory tract infections, most notably *Ps. aeruginosa*. The result of the second category is the chronic progressive disease normally associated with CF; obstructive airway disease leading to bronchiectasis and deterioration of the airways as described previously.

The third group however, differs greatly from other CF cases. These patients undergo a rapid decline with a necrotising pneumonia, and in some cases septicaemia, a rare occurrence in CF. The progress of this group begins with recurrent fevers, leucocytosis and progressive alveolar infiltration seen by x-rays. This is then followed by acute necrotising disease. Microabscesses are formed in the lung giving a 'honeycomb-like' appearance and may be accompanied by septicaemia. Formation of the abscesses leads to a rapid decline and death (Tomashefski *et al* 1988).

This progression has been reported by many groups (including Thomassen *et al* 1985, Isles *et al* 1985, Simmonds *et al* 1990). The first recorded death due to *B. cepacia* infection in a British CF patient, a nine-year-old girl, followed this pattern.

In some ways the pattern of acute pneumonia and septicaemia resembles meliodosis, caused by the closely related *B. pseudomallei*. This was observed by Dailey and Berner in 1968. They made the comparison from a case of acute *B. cepacia* pneumonia in a non-CF patient. The main difference between meliodosis and the *B. cepacia*-associated

illness is that in meliodosis necrotising granulomata are seen. This is not the case in the pneumonia found in CF patients.

1.3.2 Virulence factors and immune evasion

The knowledge of the role of virulence factors is rather limited in comparison to *Ps. aeruginosa* (Nelson *et al* 1994) Indications based on studies using animal models indicate that *B. cepacia* is less virulent than *Ps. aeruginosa*.

Factors involved in binding to respiratory epithelia

The ability to adhere to host mucosal and epithelial surfaces is important in the establishment of lung disease. In *B. cepacia* only a few potential adhesins have been described, with work centred upon the role of fimbrae in adhesion. Around 60 % of *B. cepacia* strains have been found to have peritrichous fimbrae, whilst other strains have polar fimbriae. A 16 kDa protein similar to that of other bacterial species has been identified as the fimbral sub-unit with a role in adhesion. However this protein displays neither little cross-reactivity with monoclonal antibodies against *Ps. aeruginosa* pilin protein nor any homology with a *Ps. aeruginosa* pilin protein gene DNA probe. *In vitro* studies have shown fimbrae to increase binding to epithelia, and also a possible synergistic effect between Ps. *aeruginosa* and *B. cepacia* has been suggested, possibly due to *Ps. aeruginosa* modifying the epithelial cell surface to allow increased *B. cepacia* adhesion (Nelson *et al* 1994).

B. cepacia isolates from CF have been demonstrated to bind to mucin from both CF and non-CF sources, though to a lesser extent than *Ps. aeruginosa*. The degree of

binding varies. Those strains showing the greatest level of binding to mucin seem to give rise to the most severe respiratory disease. This has led to speculation of the role of binding to mucin in *B. cepacia* mortality and morbidity, and to why some strains are transient within the lung and others are never lost. A 22 kDa pilin-associated protein distributed sparsely in pilated strains of *B. cepacia* has been identified as a mucin binding adhesin. Subsequently the pilin has been found to have a novel morphological structure of giant intertwined fibres. The resulting cable-like structure lead to it being termed Cable Pilin (Cbl). The major 17 kDa subunit gene (*cbl A*), has been sequenced (Sajjan *et al* 1995)

Extracellular virulence factors and siderophores

B. cepacia strains may express at least three siderophore mediated iron transport systems including pyochelin, cepabactin and azurechelin. Siderophores compete for iron with host iron-binding proteins and may be linked to the ability of bacteria to establish and maintain infection. In *B. cepacia* it has been postulated that siderophores aid dissemination and inflammation in the lungs. The production of pyochelin can be correlated with morbidity and mortality in CF patients, however it is only found in around half of clinical strains (Nelson *et al* 1994).

B. cepacia produces a number of extracellular factors . They appear to be more frequently found in clinical strains. Amongst these is a 34 kDa proteinase which can break down a number of proteins. (McKevitt *et al* 1989). Intratracheal instillation of a purified form of the protein into rat lungs gives rise to a bronchopneumonia with polymorphonuclear cell infiltration and exudation into the airways. The protein also

produces an immune response, however this is not protective against subsequent infection with *B. cepacia.*

B. cepacia isolates have been found to produce lipolytic activity . This may be involved in reducing the activity of host phagocytic cells. Rat pulmonary macrophages pre-incubated with lipase, or in the presence of lipase, have a significantly lower level of phagocytosis. In addition several other extracellular factors including haemolysins and catalases are known to be produced. (Nelson *et al* 1994)

Cell surface factors

Proteins and lipopolysaccharide have both been described on the cell surface of *B. cepacia.* Both rough (R-LPS) and smooth (S-LPS) lipopolysaccharide phenotypes have been isolated from CF patients, whilst other clinical and environmental isolates are predominately of S-LPS phenotype. The R-LPS phenotype gives rise to dry colonies when plated. Serum antibodies to *B. cepacia* lipopolysaccharide from CF patients show little cross-reactivity with *Ps. aeruginosa* lipopolysaccharide indicating differences in structure and composition. Initial analysis seemed to indicate the absence of 3-deoxy-D-manno-2-octulsonic acid (KDO) in *B. cepacia* strains, however Straus and co-workers (Straus *et al* 1989) indicated its presence in two out of ten clinical isolates. KDO is believed to play an important role in toxicity and subsequent studies have since confirmed its presence in *B. cepacia* (Nelson *et al* 1994). The lipopolysaccharide differs in composition from that of *Ps. aeruginosa* in having lower levels of phosphorous but higher levels of heptose. The major saccharide components are glucose and rhamnose.

B. cepacia lipopolysaccharide extracts have been found to be toxic to mice. A complex of surface carbohydrate antigens, lipopolysaccharide and protein was found to be toxic (Straus *et al* 1989), with the lipopolysaccharide component seeming to confer the toxicity. It has been proposed that this complex is responsible for the tissue necrosis and lethality associated with *B. cepacia* pneumonia.

Exopolysaccharide production

Unlike *Ps. aeruginosa* there is little evidence to indicate the production of alginate by *B. cepacia*. Polymerase Chain Reaction (PCR) studies using primers from the *algD* gene of *Ps. aeruginosa* have indicated the absence of the gene for GDP mannose dehydrogenase, essential for alginate production, indicating the absence of alginate in *B. cepacia* (Nelson 1994). This is supported by earlier evidence where an exopolysaccharide was detected but not alginate. (Sage *et al* 1990). This group found the polysaccharide to consist of galactose, glucose, mannose, glucuronic acid and rhamnose with a little uronic acid but no mannuronic or guluronic acid. It was found to be produced by glucose dehydrogenase deficient mutants (Gcd^-) and may lead to mucoid-type colonies. No correlation between exopolysaccharide production and clinical disease has been found.

In contrast to the above evidence Straus and co-workers (1989) investigating the toxicity of the surface antigen complex, found in one *B. cepacia* strain, 61g, that the carbohydrate component included an alginate-like compound containing 72 % guluronic acid, with 1.75 % acetylation of as yet unknown signifigance.

Immune evasion

B. cepacia seems able to resist a relatively strong humoral immune response. A wide range of sensitivity to serum antibodies and resistance to killing by polymorphonuclear cells has been described. This may be due to phenotypic differences in the cell envelope. As described previously, patients with chronic granulomatous disease (CGD), a defect in phagocytic cells' oxidative killing mechanism, are particularly susceptible to *B. cepacia* infection. This appears to be a result of *B. cepacia's* ability to resist neutrophil mediated non-oxidative killing (Nelson *et al* 1994).

The high levels of immunity displayed by *B. cepacia* have led to speculation regarding the relative ease of the bacteria's ability to survive within the CF lung. Recent evidence that *B. cepacia* can invade respiratory epithelial cells *in vitro* suggests that the bacteria has the ability to invade and survive within lung epithelium (Burns *et al* 1996a).

1.4 Isolation and identification of *B. cepacia*

1.4.1 Isolation of *B. cepacia*

Prior to the mid 1980's the study of *B. cepacia* was hindered considerably by lack of a suitable isolation media. In the mid 1980s media for the isolation for *B. cepacia* from CF patients (Gilligan *et al* 1985, Welch *et al* 1987) and the environment were developed (Wu and Thompson 1984, Hagedorn *et al* 1987).

Media developed for the isolation of *B. cepacia* from CF patients' sputum rely to an extent on the intrinsic antimicrobial resistance of the species. Prior to the development of these media, isolation was primarily conducted on sheep blood agar or MacConkey agar followed by identification. *Ps. cepacia* (PC) medium (Gilligan *et al* 1985) consists of inorganic salts, 0.5 % pyruvate and 0.1 % protease peptone as nutritive components, with 0.0001 % crystal violet, 0.15 % bile salts, 100 $\mu g\ ml^{-1}$ ticaricillin and 300 $U\ ml^{-1}$ of polymixin B as selective agents. OFPBL agar (Welch *et al* 1987) is a supplemented oxidation-fermentation (OF) agar. To produce the OFPBL agar, lactose, polymixin B (300 $U\ ml^{-1}$) and bacitracin (0.2 $U\ ml^{-1}$) are added to OF medium.

On PC medium 38 of 50 *B. cepacia* stock isolates grew after 48 hours of incubation compared with none on MacConkey agar (Gilligan *et al* 1985). In clinical testing 35 out of 169 CF respiratory secretions were found to contain *B. cepacia* using PC medium as compared with 21 using MacConkey agar. The PC medium inhibited 112 of 124 streak isolates of various other species found in CF respiratory secretions and only 6 of 221 other potential pathogenic isolates grew (two *Ps. aeruginosa*, one

Serratia marcescens, two molds and a yeast). *B. cepacia* often produces pink colonies on PC medium. OFPBL was also found effective in clinical trials yielding *B. cepacia* in 58 of 725 samples as compared with 19 by MacConkey or sheep blood. Acidification of the medium by *B. cepacia* produces a yellow colour which differentiates it from yeasts, though acidification by *B. gladioli* and Ps *aeruginosa* were reported.

Both media were subsequently used in the study of *B. cepacia* and in diagnostic use by microbiology laboratories. PC medium has been produced commercially by Mast diagnostics, and is widely used in UK laboratories. Growth of drug resistant *Pseudomonas* species, *Candida* spp, *Comomonas acidovorans* and *Stenotrophomonas maltophilia* may be problematical and lead to misidentification (Burdge *et al* 1995, Henry *et al* 1997). To counter such problems BCSA (*B. cepacia* selective agar) has been recently developed (Henry *et al* 1997). This medium contains, sodium chloride, sucrose, lactose, yeast extract, trypticase peptone, crystal violet, phenol red, polymixin B (600 U) and gentamicin (10 $\mu g\ ml^{-1}$). Vancomycin at 2.5$\mu g\ ml^{-1}$ may also be added. BCSA appears to have similar sensitivity to OFPBL or PC detecting 190 of 191 CF *B. cepacia* isolates (99.5%), compared with 100 % for OFBL and 94 % for PC, but with fewer non-*B. cepacia* isolates growing; 10 of 189 (5.3 %) Gram positive and Gram negative organisms grew on BCSA without vancomycin, compared to 13.8 % false positives on PC and 19.6 % on OFPBL. The addition of vancomycin reduced the rate of false positives to 3.7 % without affecting the growth of *B. cepacia* isolates. Growth on BCSA also appears to be more rapid and could allow quicker identification.

Identification of *B. cepacia* in water samples is important in the pharmaceutical industry. The versatile nature of the organism means it is able to grow in pharmaceutical compounds if introduced by a contaminated water source. Wu and Thompson (1984) developed a media consisting of plate count agar supplemented with 9-chloro-9 (4-diethylaminophenyl) -10-phenylacridan, a quinacrine compound inhibiting DNA replication, at 1 $\mu g\ ml^{-1}$ and polymixin B at 75 $\mu g\ ml^{-1}$ The medium inhibited growth of all tested bacteria except *B. cepacia* and *Serratia marcescens*, which can be differentiated by morphology and oxidase activity.

Hagedorn *et al* (1987) developed a medium for isolation of *B. cepacia* from soils. TB-T combined trypan blue (TB) and tetracycline (T) with mineral salts, glucose as a carbon source and L-asparagine as a nitrogen source along with crystal violet and/or nystatin to inhibit fungal growth, at pH 5.5, platings from soil cultured 300 isolates, 216 of which were identified as *B. cepacia* (72 %). The remainder of organisms (28%) identified mainly as *Serratia* species could be separated from *B. cepacia* by anaerobic glucose fermentation. The media is not as selective as clinical media but allows recovery of *B. cepacia* from soil at low dilutions.

1.4.2 Identification

A number of factors make the identification of *B. cepacia* difficult. Maintenance of cultures is difficult as *B. cepacia* is only viable for a short time on nutritive agars and many isolates do not survive at 4°C requiring maintenance at –70°C (Gilardi 1983, Govan and Harris 1985, Pitt and Govan 1993). Gilardi (1983) described the use of biochemical tests and sugar acidification/fermentation for identification. The use of

biochemical and nutritive tests has proved difficult due to variable properties in key tests. Hagedorn *et al* (1987) describe both oxidase negative and oxidase positive isolates of *B. cepacia*, whilst two identification methods describe the organism as oxidase negative in one case, positive in the other. Pitt and Govan (1993) describe the difficulty posed by asaccharolytic strains.

In UK clinical laboratories *B. cepacia* is usually identified by growth on Mast PC selective medium followed by confirmation using BioMerieux API 20 NE identification strips (Pitt and Govan 1993). In the US a number of commercial identification methods have been applied notably API Rapid NFT, however problems with misidentification due to either growth on selective media or confirmation with commercial methods have been noted (Burdge *et al* 1995, Kiska *et al* 1996). Kiska *et al* (1996) assessed the accuracy of four commercial systems: Rap ID NF plus, API Rapid NFT, Vitek AUTO MICROBIC system and Remel Uni N/F Tek and N/F Screen, in typing *B. cepacia* and other Gram negative non-fermenting bacilli from CF patients. Accuracy of *B. cepacia* typing ranged from 46 to 86 % and 57 to 80 % for other non fermenting bacteria. Various *Pseudomonas* species and other *Burkholderia* species were misidentified as *B. cepacia*, suggesting a requirement for further confirmatory tests.

Fatty Acid Methyl Ester (FAME) profiling based upon the fatty acid composition of cells by gas chromatography is a highly accurate method of typing (Leff *et al* 1995), especially in environmental strains. However the technique is rather specialised for a standard laboratory. A number of molecular techniques based upon ribosomal RNA (r

RNA) genes have been described for identification and typing of *B. cepacia*. Typing is discussed in detail in the following section. Leff *et al* (1995) described the identification of aquatic *B. cepacia* using hybridisation with species specific 16S and 23S rRNA gene probes. This proved more accurate than API test strips or selective media and more efficient than FAME profiling.

Amplification of 16S rRNA DNA sequences by polymerase chain reaction (PCR) followed by DNA hybridisation has been used to identify *B. cepacia* and *Ps. aeruginosa* directly from patients' sputum (O'Callaghan *et al* 1994). PCR identification based on primer sequences from 16S-23S rRNA gene internal transcribed spacers (ITS) for *B. cepacia* and other *Burkholderia* species and pseudomonads has also been tried, along with tRNAs and structural genes (Tyler *et al* 1995). The prokaryote rRNA operon (*rrn*) contains genes from 16S, 23S and 5S rRNA. Multiple rrn loci frequently occur with differences in repetition, arrangement and linkage of the loci. Identification can be made on the basis of the differences in ITS length polymorphisms. PCR amplification using primers based on ITS sequences should therefore identify species on basis of inter-species polymorphisms. This approach was successful for several *Burkholderia* and *Pseudomonas* species, however 2 of the 3 *B. cepacia* reference strains and many of 103 tested clinical isolates were not detected using primers obtained from sequence data obtained from strain ATCC 25609. Alternate primers were designed using other ITS sequences, again these were specific for *B. cepacia* but the method proved incapable of detecting a range of isolates leading to the abandoning of ITS sequence primers. Primers targeting 23S rRNA genes

directly were developed. These were successful, being specific for *B. cepacia* and detecting all isolates tested.

1.4.3 Typing

Typing is needed for epidemiological studies and also to assess prognosis by detection of epidemic strains. Several methods have been used to type *B. cepacia* including FAME analysis, serological typing, bacteriocin typing, ribotyping, multilocus enzyme electrophoresis and pulsed field gel electrophoresis (PFGE) analysis (Rabkin *et al* 1989, Pitt and Govan 1993, Wilkinson and Pitt 1995a). An international collaborative study was conducted in the late 1980's to assess several potential typing systems (Rabkin *et al* 1989). The survey found serotyping and ribotyping to be specific and sensitive. Typing by antimicrobial susceptibility and biotyping, such as use of API strips to be both less specific and sensitive. Bacteriocin typing was found to be the most sensitive method, though much variation in production and sensitivity was found between isolates. The use of bacteriocin typing is based upon production and sensitivity to bacteriocins. Bacteriocins are antimicrobials that affect related species or other strains of the same species. Bacteriocin typing was first applied in *B. cepacia* by Gonzalez and Vidaver (1979). Govan and Harris (1985) developed a method using a multipoint inoculator to inoculate *B. cepacia* or *Pseudomonas* test strains onto agar plates. These are grown for 5 hours, then test isolates are chloroform killed. The *B. cepacia* or *Pseudomonas* indicator is added in an agar overlay and incubated for 18 hours. Bacteriocin production or sensitivity is indicated by an inhibition zone. Inhibition patterns allow typing of isolates into groups based on production and sensitivity.

Serological typing using agglutination to detect heat stable antigens has been used by several groups (Wilkinson and Pitt 1995a). Initially five serogroups were defined and subsequent studies found seven O and five H antigens, other groups found five and ten different antigen groups.

Ribotyping has been employed as a consistent and accurate typing method (Rabkin *et al* 1989). The process involves digestion of chromosomal DNA with a restriction endonuclease; usually *Eco*R1 followed by Southern blot hybridisation with labelled *E coli* RNA. Typing is achieved by restriction fragment length polymorphisms (RFLP) between isolates leading to variances in number and position of hybridisation bands. At least 55 different ribotype patterns have been observed in UK CF isolates (Wilkinson and Pitt 1995a). More detailed analysis may be conducted by external ribotyping (Rozee *et al* 1994). This utilises typing by digesting with four restriction enzymes. This group have also employed multilocus enzyme electrophoresis (MLEE) (Rozee *et al* 1994, Johnson *et al* 1994) as a typing method. This involves analysing the electrophoretic mobilities of 14 enzymes. The use of MLEE typed 83 mainly CF isolates into 20 electropherotypes (ET's). These approaches allowed genetic linkage and relationships to be investigated. One type ET12 was found to be responsible for an epidemic of fatalities in the UK and Canada. Extended ribotyping allowed detailed analysis of ET12 clones in the UK and Canada. Strains from London, and particularly Manchester and Ontario were closely related, suggesting a common origin. ET12 clones from Edinburgh and Belfast were found to be more distantly related. More recently use of PCR methods similar to those developed for identification have been applied to typing and have the advantage of not being as complex or labour intensive as

conventional ribotyping. PCR ribotyping, as the technique has been dubbed, was first used to type *B. cepacia* by Kostman *et al* (1992) by differences in amplification patterns of the intergenic spacer between 16S rRNA and 23S rRNA genes. As described previously most bacteria have multiple copies of the rrn operon (rRNA operon) and also differences in the length and sequence of 16S-23s intergenic spacer regions between operons within the same genome and between the operons in a bacterial species may occur. Amplification of the spacer regions will therefore give rise to multiple PCR products. Different lengths and numbers and PCR products could arise due to the polymorphisms between strains, so electrophoresis of the PCR products may yield different patterns between species strains (Kostman *et al* 1992). If isolates are of the same strain an identical pattern would be obtained i.e. are of the same ribotype; differing strains would have a different pattern or ribotype. The concordance of PCR ribotyping with conventional ribotyping was found to be 93% for 90 isolates from seven well characterised outbreaks of *B. cepacia* infection including in the UK and Denmark (Ryley *et al* 1995, Ryley *et al* 1996). Amplification with primers to the 16S-23S rRNA genes was followed by a *Taq* I restriction digest to give distinct ribotyping patterns. The use of the *Taq* I digest gives rise to more easily discernible patterns. Seven distinct patterns of ribotypes were found in 16 isolates from CF patients in Wales (Ryley 1995). Seven isolates were found to be of one ribotype pattern, four others showed a distinct pattern and two another. None were found to be of the ET12 epidemic strain. In Denmark 12 isolates were investigated with 8 different ribotypes found by this method. DNA finger printing by Pulsed Field Gel Electrophoresis (PFGE) has been applied to typing of *B. cepacia* strains (Anderson *et al* 1991). This technique involves embedding bacteria cells in agarose in which the

cells are lysed with sodium lauroyl sarcosine-proteinase K buffer. Embedded chromosomal DNA is then digested with a rare cutting restriction enzyme. The digested DNA is then separated by PFGE. A DNA fingerprint is so obtained for the isolate tested. A number of enzymes may be used to obtain such fingerprints. Comparison of fingerprints may be made between isolates to characterise them. The technique has also been called macro-restriction analysis. In the UK analysis after digestion with *Xba* I and *Spe* I has been utilised to type 366 isolates (Pitt *et al* 1996). More than 50 different patterns were found, with 68 patients found to harbour one strain termed ribotype 1 at 8 different UK CF centres.

Randomly amplified polymorphic DNA typing or RAPD analysis has recently been applied to typing of *B. cepacia* (Mahenthiralingam *et al* 1996a, 1996b). The method uses PCR primers from DNA extracted from *B. cepacia* ATCC 25416 (Initially a range of 100 random primers were used, those that were able to detect polymorphisms, 8 in this case, were able to give fingerprints). The selected primers were used to randomly amplify DNA by PCR, each primer producing a fingerprint of 5 to 20 bands of 100 bp to 5 KB. The fingerprints produced can be analysed to type strains on basis of similarity and difference. The technique was able to type 627 *B. cepacia* isolates previously typed by PFGE or conventional ribotyping with greater sensitivity than PCR ribotyping. A conserved DNA marker of 1.4 Kb was found by RAPD analysis of epidemic strains (Mahenthiralingam *et al* 1997). The marker was found in strains from seven CF epidemic strain outbreaks but not in non-epidemic strains. The marker was designated as *B. cepacia* epidemic strain marker (BCESM). Only one of the seven

strains was found to contain the cable pilin subunit gene (*cblA*) found in an epidemic strain from UK and Canada.

1.5 Epidemiology of *B. cepacia* in CF

Studies in the early to mid 1980's recognised the importance of *B. cepacia* in CF. Several factors were suggested to increase the rank of *B. cepacia* colonisation (Reviewed by Tablan *et al* 1987a and b):

a) Existing severe underlying CF lung disease,

b) A *B. cepacia* colonised CF sibling,

c) Increased age,

d) Prior treatment with aminoglycoside antibiotics was all implicated in colonisation and morbidity. However because of the lack of a selective medium and adequate typing techniques made the identification of source of infection and transmission difficult. Development of specific isolation media improved the isolation of *B. cepacia*. The subsequent development of typing techniques has allowed more effective study.

1.5.1 Person-to-person transmission

Due to the the development if the above methods strong evidence of person-to-person transmission of *B. cepacia* amongst CF patients has been obtained. Evidence of person-to-person transmission has been described in both the UK and N. America. Initial evidence was reported in the US in 1990 (Li Puma *et al* 1990, 1994), with subsequent evidence in Wales (Millar Jones *et al* 1992), Scotland (Govan *et al* 1991, Govan *et al* 1993), England (Smith *et al* 1993) and Canada (John *et al* 1994). There is also substantial evidence that social contact between patients has led to transmission. Smith *et al* (1993) reported evidence of incidence of *B. cepacia* in the East Birmingham Hospital adult cystic fibrosis unit despite segregation of *B. cepacia*

positive and *B. cepacia* negative patients within the hospital. Subsequent questioning of patients' social activity revealed considerable social contact with infected patients, even a case of intimate contact. Govan and co-workers (1993) found similar evidence, including transmission between patients in British CF centres and patients attending Canadian CF summer camps. Studies in Canada (John *et al* 1994) have produced similar evidence whether transmission indirect via droplet, or via intimate contact with fomites such as drinking cups is unclear. Govan *et al* (1996) also described the isolation of B. *cepacia* from drinking cups, sputum containers and gloves of doctors attending *B. cepacia* colonised patients. There is also evidence of airborne dissemination of *B. cepacia* in adult CF patients (Humphreys *et al* 1994). Surface and air sampling onto selective medium recovered *B. cepacia* in 5/6 patients.

The evidence of person-to-person transmission has led to segregation polices in many hospitals to avoid possible transmission of *B. cepacia* from infected to non-infected CF patients, and the prevention of *B. cepacia* colonised patients attending CF summer camps in N. America and Europe. In the UK the CF trust requires *B. cepacia* colonised patients not to attend social events and meetings. Such policies have serious social implications for colonised patients but seem to be necessary to prevent further infection. Reports have also linked home-use nebulizers with *B. cepacia* and other Gram negative bacterial infections in CF (Hutchinson *et al* 1996). 3 of 35 nebulizers tested were found to harbour *B. cepacia*. Most of the patients in this study were receiving colistin or gentamicin therapy, this being a suggested predisposition for *B. cepacia* infection. The study also suggested the requirement for good cleaning, drying

and maintenance of nebulizers to avoid their becoming potential reservoirs of *B. cepacia* or other Gram negative infection.

Ribotype 1 appears to be the main epidemic strain within the UK and Ireland and has been found in 68 patients at several different centres indicating cross-colonisation (Pitt *et al*1996) However the levels of antimicrobial resistance within ribotype 1 appear to vary greatly. Comparative study of *B. cepacia* infection by multilocus enzyme electrophoresis and ribotyping of Canadian and UK epidemic strains along with other CF isolates, non CF nosocomial infections and environmental isolates revealed 20 electrophoretic types (ET's) or clones. The strains causing the *B. cepacia* epidemic amongst CF patientswere found to be one clone (ET12). Ribotype 1 falls into this clone. Isolates of ET12 clones have been found to vary in their enzyme ribotypes. The variability of ribotypes may be associated with the highly virulent strain leading to poor prognosis (Rozee *et al* 1994). Another Canadian CF clone, ET16, has much lower variability and is associated with a better progress. Similarly between some of the Canadian and British strains have led to the suggestion that the epidemic strain was brought to the UK by patients attending CF summer camps in Canada. Data on the virulence of ribotype 1 (Pitt *et al* 1996) is patchy but its' clinical presentation in the UK seems to vary from asymptomatic to highly virulent but appears to be highly transmissible. As described previously the exact factors determining *B. cepacia* virulence in CF are unknown, however Edinburgh CF strains of ribotype 1 display a level of mucin adherence six times greater than other strains.

More recent evidence has indicated that some *B. cepacia* strains display low transmissibility as transmission only occurred in 3 of 8 sets of CF siblings and evidence

of person to person transmission occurred in only 16.7% of unrelated cases of *B. cepacia* infection (Cazzola *et al* 1996), whilst a number of sporadic cases appeared to have an environmental source. Despite segregation policies in hospital person to person transmission appears to occur. This appears to be due to social contact outside hospital. Such transmission appears to be either by direct contact or by indirect contact (Cazzola *et al* 1996, Amalfitano *et al* 1996). The ability of *B. cepacia* to survive in respiratory droplets and environmental surfaces for long periods of time is significant in terms of indirect person-to-person transmission (Drabick *et al* 1996). It has also been suggested that *B. cepacia* may be disseminated into the environment during physiotherapy (Ensor *et al* 1996). This reaffirms the need for segregation policies in hospitals and also guidelines given to patients in Europe and N. America to avoid activities that increase the spread of *B. cepacia*. The guidelines issued in Canada for example suggest avoiding close social contact such as kissing, segregation in hospitals, not sharing respiratory therapy equipment, toothbrushes or handkerchiefs or sharing a room during physiotherapy or exercise. Good personal hygiene and care of equipment such as nebulizers is also recommended to avoid infection from the environment (Canadian Cystic Fibrosis Foundation 1995).

1.5.2 Non CF infections

In the US a significant increase in non-CF nosocomial infections has been observed in recent years (Pegues *et al* 1996). In the period 1980 to 1985, 3.5 per 100,000 medical admissions and 3.3 per 100 000 surgical admissions to US hospitals were found to have acquired *B. cepacia* infections, 31% of the cases involving lower respiratory tract infections.

In a recently described nosocomial outbreak (Pegues *et al* 1996) the majority of infected patients had undergone mechanical ventilation in the intensive care unit. These patients were more likely to have undergone 2 or more days of ventilation and multiple intubations than the non-*B. cepacia* infected control group. In addition the infected patients were significantly more likely to have undergone nebulised therapy or a course of antimicrobial therapy with cephalosporin and aminoglycoside. Other reports have linked the use of nebuliser therapy with nosocomial outbreaks of *B. cepacia* and other bacteria; one of the more recent reports linking nebulised albuterol therapy with a *B. cepacia* outbreak (Hutchinson *et al* 1996). These reports suggest the need for good practice and disinfection to avoid nebulizers and other respiratory equipment being the source of non-CF nosocomial outbreaks

1.6. *Burkholderia cepacia* in the environment

There are a number of areas in which *B. cepacia* is of interest in the environment; as a potential reservoir of infection (as described previously), as a phytopathogen, as an agent of bioremediation particularly due to its versatile catabolic properties and as an agent of biocontrol against phytopathogenic fungi damaging crops.

1.6.1 *B. cepacia* as a phytopathogen

B. cepacia was first described as a phytopathogen in 1950 by Burkholder. He described a rot affecting the outer storage scales of onion (*Allium cepa*) causing maceration of tissue accompanied by a yellow, slimy appearance and a distinct sour 'vinegar-like' odour. The disease had been termed 'sour skin' or 'slippery skin' onion rot by onion growers in New York State, USA, and had been attributed to be the cause of the loss of up to 20% of the onion crop. Burkholder isolated the causative agent, a Gram negative rod-shaped bacterium, and named it *Ps. cepacia*.

B. cepacia, along with *B. gladioli* and *Erwinia herbicola* are known to be the causative agents of bacterial soft rot of onions. *B. cepacia* is described as causing a rot of onion bulb scales, usually occurring near maturity or sometimes in storage. It affects most *Allium* species but is not thought to be a strongly invasive pathogen, attacking mainly weakened plants or those nearing maturity (Bradbury 1986). It has also been found to cause rots in maize, beans, peas and tomatoes if inoculated into these plants. *B. gladioli* p.v. *allicola* causes a rot with softening and dark discoloration of outer scales, whilst *E. herbicola* is primarily a rot of onion storage. Both *B. cepacia* and *B.*

gladioli produce pectic enzymes, *Erwinia* does not. It is thought that it is this pectinolytic activity which causes the maceration of onion tissue by these bacteria.

The pathogenicity of *B. cepacia* strains may be assessed by conducting host tests, either by the inoculation of onion tissue slices or whole onions. Such tests have been used to investigate the pathogenicity of clinical and environmental strains. One study showed clinical strains to have little or no ability to macerate onion tissue, or have little pectinolytic activity (Gonzalez and Vidaver 1979). Pathogenicity to onion and pectinolytic activity were used as part of the criteria in the division of *B. cepacia* into four groups or phenoms(Yohalem and Lorbeer 1994). Differences have been found both between and within clinical and environmental strains, but generally indicated that clinical strains are poor plant pathogens. However, onion maceration by clinical strains, including the epidemic strain associated with CF, has subsequently been demonstrated (Butler *et al* 1995).

Ps. aeruginosa has been reported to cause a *Burkholderia*-like onion rot (Cother *et al* 1976), though rot only seems to occur under a very specific set of growth and storage conditions. It does not cause rot *in vitro*, and at most could be considered a 'quasi-pathogen' (Cother *et al* 1976).

1.6.2 Production of antibiotics and biocontrol by *B. cepacia*

B. cepacia has been found to produce a number of antibiotics including pyrrolnitrin, altercidins, cepacin and xylocandins (Homma *et al* 1989). Although cepacin and xylocandins have been investigated for potential therapeutic use, it is in the field of

biocontrol, the biological control of plant pathogens, that such compounds would appear to be most effective.

B. cepacia antibiotics and their potential therapeutic use

B. cepacia ATCC 29356 was found to produce two acetylenic antibiotics , cepacin A and B (Parker *et al* 1984). Cepacin A has strong activity against *Staphylococci* (a minimum inhibitory concentration (MIC) of 0.2 $\mu g\ ml^{-1}$) and weaker activity against *Streptococci.* Cepacin B has some activity against Gram negative bacteria and excellent activity against *Staphylococci* (MIC < 0.05 $\mu g\ ml^{-1}$). Xylocandins, a complex of novel peptides isolated from strain ATCC 39277 show considerable activity against a range of *Candida* species and other dermatophytic fungi *in vitro* (Meyers *et al* 1987). However the lack of efficacy of xylocandin in *in vivo* studies in mice, and the relative toxicity of cepacin have curtailed any further development as therapeutic antimicrobials.

B. cepacia as an agent of biocontrol

The failure of control measures against plant pathogenic fungi is largely due to the development of resistance to chemical fungicides. This resistance along with public concern over the safety of such chemicals, has lead to an interest in the use of biological methods in controlling phytopathogens. This involves the protection of fruit and vegetable crops either by the use of organisms antagonistic to pathogens, or by the compounds produced that cause the antagonism. *B. cepacia* has been reported to suppress the growth of many phytopathogenic fungi including soil-borne diseases such as *Fusarium sp.* wilt of tomato, 'damping-off' of radish and *Verticullum sp.* wilt of

aubergine (Homma *et al* 1989). This suppression was achieved either by using *B. cepacia* as a seed-coating or a root-dip. Antagonism of *Bipolaris maydis*, the causative agent of leaf blight in maize by *B. cepacia* has also been recorded (Sleesman and Leben 1976).

Study of the compounds that suppress pathogen growth produced by *B. cepacia* RB 42S showed that pyrollnitrin and two pseudane derivatives (HMQ and NMQ) were the main suppressive compounds (Homma *et al* 1989). These compounds were found to have activity against a range of phytopathogenic fungi including *Verticullum chahiae, Rhizoctoniia solani, Pythium ultimum* and *Fusarium oxysporium*, but showed little antibacterial activity. *B. cepacia* has also been described as an antagonist of Blue mold (*Penicillium expansum*) and Gray mold (*Botyris anerea*) of apples and pears (Janisiewicz and Roitman 1988). Pyrrolnitrin, isolated from a *B. cepacia* strain growing on apple leaves, was used to treat apples and pears *in vitro*. The treated fruit displayed no fungal lesions or rot when inoculated with either *B. anerea* or *P. expansum*, whilst untreated fruit developed considerable rot. *Pseudomonas fluorescens* has also been found to produce pyrrolnitrin and has been used to protect cotton seedlings from *Rhizoctonia solani* rot. Altercidins, a type of phenazine antibiotic, are also produced by *B. cepacia*. This type of antibiotic produced by *Ps. fluorescens* has been shown to suppress a fungal disease of wheat caused by *Gaeumannomyces gramnis* (Thomashow and Weller 1988).

More recent research has demonstrated the potential of *B. cepacia* as a suppressor of phytopathogenic fungi in maize (Jayasawal *et al* 1990. Hebbar *et al* 1992). Maize is

one of the most important cereal crops worldwide, but is susceptible to soil-borne fungi. *B. cepacia* is antagonistic to a number of these fungi including *Trichodema*, *Fusarium* and *Aspergillus* species. *B. cepacia* seems to be particularly effective as a biocontrol agent in maize, as seed inoculation with a small amount of bacteria leads to rapid colonisation of the roots and the rhizosphere. This is due to its' high growth rate, production of extracellular enzymes and its' ability to utilise a wide range of plant exudates (Hebbar *et al* 1992). *B. cepacia* also suppresses the sunflower (*Helianthus annus*) phytopathogen *Sclerotina sclerotionum* which causes root rot and wilt of the plant (McLoughlin *et al* 1992). Inhibition of the fungus was found to be as a result of pyrrolnitrin production, along with production of the related compounds aminopyrrolnitrin and monochloroaminopyrrolnitrin.

1.6.3 *B. cepacia*, biodegradation and bioremediation

The ability of *B. cepacia* to utilise a wide range of carbon sources has been known for some time. The contamination and growth in disinfectants (Holmes 1986) and the ability to utilise penicillin G as a sole carbon source (Beckman and Lessie 1979) are examples of the extraordinary metabolic diversity of the organism. The previous synonym *Pseudomonas multivorans* suggests this ability more clearly than the present nomenclature. The ability to catabolise a range of compounds recalcitrant to breakdown including chlorinated aromatic compounds, notably 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T) by the *B. cepacia* strain AC1100(Kilbane *et al* 1982), have earmarked *B. cepacia* as a potential agent of bioremediation (Govan *et al* 1996). 2, 4, 5-T has been widely used a a herbicide, particularly as the major component of the defoliant Agent Orange used extensively by US forces in the Vietnam

War and has been demonstrated to have severe toxicological effects as a carcinogen and teratogen (Kilbane *et al* 1982). It has been suggested that this metabolic diversity may be due to the high degree of plasticity within the *B. cepacia* genome (Lessie *et al* 1996). The extensive array of insertion elements promote genetic rearrangement and promote expression of recombinant genes. This has been suggested to have played a role in the evolution of novel catabolic processes, including that of 2, 4, 5-T (Haugland *et al* 1991).

Degradation of phenoxyacetate herbicides such as 2, 4, 5-T and 2, 4-dichlorophenoxyacetate (2, 4-D) has been well described in *B. cepacia* and the genetics of the degradation pathways elucidated, such as the *tft* genes in 2, 4,5-T degradation by AC1100 (Daubaras *et al* 1996) Frequently these pathways are plasmid encoded. Bhat *et al* (1994)found a 90 kb plasmid they named pMAB 1 in wild type *B. cepacia* CSV 90 encoding the ability to use 2,4 dichlorophenoxyacetate 2,4-D and 2 methyl-4 - chlorophenoxyacetate as sole carbon source. 2,4-D$^-$ mutants were found not to contain this plasmid or contain a smaller 70 kb plasmid termed pMAB 2. Cloning of a 10 kb fragment absent from pMAB 2 but in pMAB1 into *E. coli* resulted in the production of a 3,5 dichlorocatechol dioxygenase, a likely component of a degradative pathway. Xia *et al* (1998) described a 105 kb plasmid,pIJB1, possesing a 41 kb transposon, Tn*5530*, encoding for 2, 4-D degradation. Haak *et al* (1995)described a 70 kb plasmid, designated pBAH 1, encoding for a 2 component 2-halobenzoate 1,2 dioxygenase from *B. cepacia* 2CBS. Toluene and trichloroethylene, a common groundwater pollutant, can be catabolised by *B. cepacia*G4 (Folsom *et al* 1990). This pathway is encoded by a 108 kb plasmid named TOM (Shields *et al* 1995).

B. cepacia strain RKJ 200 is capable of utilising *p*-nitrophenol (PNP) as the sole carbon and energy source (Prakash *et al* 1996). A 5 kb plasmid carries the genes encoding for degradation via hydroquinone and the beta-ketoadipate pathway, along with resistance to inorganic zinc ions. *B. cepacia* Pc 701 catabolises 4-methylphthalate (Saint and Romas 1996). Phthalate wastes are produced and released by the paint, plastics and paper industries and are of concern due to their potential toxicity. *B. cepacia* is currently being suggested as a potential bioremediator of these wastes. Degradation is thought to proceed via a phthalate dioxygenase pathway. Pc 701 contains a plasmid pMOP. This contains a gene cluster thought to encode for degradation and a protein thought to be involved in phthalate uptake.

In addition to the degradation of chlorinated and other aromatic hydrocarbons by *B. cepacia*, a mineral solubilising gene has been cloned (Babu-Khan *et al* 1995). The gene results in solubilisation of calcium phosphate by gluconic acid produced by the direct oxidation pathway of glucose,

1.7 Antibiotics and antimicrobial chemotherapy and their use in *B. cepacia* infections

1.7.1 Action of antimicrobial drugs

The action of antimicrobial drugs fall into four main groups (Russell and Chopra 1990):

1. Drugs that inhibit cell wall synthesis - penicillins, cephalosporins, vancomycin.
2. Drugs that inhibit protein synthesis - aminoglycosides, tetracyclines, chloramphenicol and erythromycin.
3. Drugs that inhibit nucleic acid synthesis - sulphonamides, trimethoprim and quinilones.
4. Drugs that inhibit membrane integrity - polymixins, iontophoric antibiotics, polyene drugs.

1.7.2 Drugs that inhibit cell wall synthesis

β-lactam antibiotics are the main group of drugs that inhibit cell wall synthesis (Franklin and Snow 1989, Russell and Chopra 1990). These are bactericidal drugs that act by preventing cross-linkage of peptidoglycan in cell wall synthesis. There are 2 main groups of β-lactams; penicillins and cephalosporins. Both share the common structure of a β-lactam ring' a 4 membered, cyclic amide ring.

The synthesis of peptidoglycan occurs in 4 stages. The first stage is the synthesis of pre-cursors in the cytoplasm. This is followed by formation of a pentapeptide. The pentapeptides are then linked to form a linear peptidoglycan polymer, which is then

cross-linked to give a strong rigid structure. The mode of action of β-lactams is based upon similarity between the β-lactam ring and the *D*-alanine *D*-alanine end of the peptidoglycan side-chain. β-lactams compete with the side chains for binding to the transpeptidases and carboxypeptidases that catalyse polymerisation of the glycan chains and cross linking. The β-lactams form stable complexes when bound to the enzymes leading to enzyme inactivation and causing termination of polymerisation and cross-linking. Intermediates of peptidoglycan will accumulate causing the activation of autolysis and destruction of the cell. The enzymes to which β-lactams bind are also known as penicillin binding proteins of PBP's.

A number of other drugs inhibit peptidoglycan synthesis. *D*-cycloserine and fosfomycin act in the early stages of synthesis to prevent pentapeptide formation. Bactitracin blocks the dephosphorylation of a lipid carrier molecule preventing transfer of pentapeptide units in the formation of the peptidoglycan polymer. Vancomycin binds to the *D*-alanine *D*-alanine terminus of the peptidoglycan precursor units preventing transglycosylation of the units and formation of the peptidoglycan polymer chain (Franklin and Snow 1989).

β-lactams are widely used in the treatment of CF respiratory infections. *Ps. aeruginosa* and *B. cepacia* are both resistant to most first generation penicillins and cephalosporins (Prince 1986). However antipseudomonal and extended spectrum penicillins such as azlocillin and piperacillin have been widely used, with most *B. cepacia* strains moderately susceptible to these drugs *in vitro* (Bhakta *et al* 1992). These drugs are usually administered parenterally, often in combination with

intravenous oral or nebulised aminoglycosides in the treatment and prophylaxis of CF lung infection with *Ps. aeruginosa* or *B. cepacia* (Kumar *et al* 1989). However *B. cepacia* almost invariably proves resistant to aminoglycosides, so such treatment is generally ineffective and somewhat controversial as it has been suggested that prophylactic treatment with aminoglycosides 'primes' the lungs for subsequent *B. cepacia* infection (Govan & Nelson 1992).

The aminothiazole cephalosporin ceftazidime is very active against both *B. cepacia* and *Ps. aeruginosa in vitro,* and has proved effective against *Ps. aeruginosa* infections in CF but unfortunately it has proved less successful against *B. cepacia in vivo*, probably due to difficulty in achieving a high enough concentration of the drug within the lungs (Gold *et al* 1983). This being one of several problems associated with antimicrobial chemotherapy of the CF lung. (Discussed in further detail later) Despite these problems ceftazidime is one of the main weapons in the therapy of *B. cepacia* infection. With around 90% of clinical isolates found to have susceptibility *in vitro* (Bhakta *et al* 1992) it is one of the most effective agents available for treatment of *B. cepacia* respiratory tract infection. It is given intravenously, often in combination with other antibiotics such as lincomycin (Simmonds *et al* 1990)

1.7.3 Drugs that inhibit protein synthesis

A number of drugs act to inhibit protein synthesis, employing several different modes of action. Aminoglycosides are a group of compounds that consist of 2 or more amino sugars linked by glycosidic bond to a central hexose core. Aminoglycosides are bactericidal and work in a number ways. Streptomycin binds to the 30s ribosomal

subunit blocking protein synthesis by preventing binding of amino acyl t-RNA. Neomycins, gentamicins, kanamycins and tobramycin bind to multiple sites on the ribosome directing 16S RNA to bind to the 30 S subunit and so inhibiting translocation of amino acids from the A to P sites on the ribosome. This then prevents elongation of the polypeptide chain (Russell and Chopra 1990).

As described previously various regimens of aminoglycoside antibiotics in combination with β-lactam antibiotics have been used in treating CF lung infections, though these are generally ineffective due to the high levels of aminoglycoside resistance displayed by *B. cepacia*. Nebulised antibiotics, i.e. delivered as an aerosol, have proved more effective than other dosage forms as they are delivered directly to the lung epithelia, with tobramycin, a drug well tolerated by both adults and children, proving effective in controlling lung infection by *Ps. aeruginosa* (Friel 1995).

Tetracyclines are broad-spectrum antibiotics that inhibit protein synthesis in 70 S bacterial ribosomes and 80 S eukaryotic ribosomes. They are bacteriostatic, acting by preventing binding of amino acyl t-RNA to the acceptor A site (Franklin and Snow 1989). Chloramphenicol is a bacteriostatic drug that binds to the 50 S subunit of 70 S ribosomes, and acts by inhibiting peptidyl transferase activity. Although chloramphenicol inhibits many strains of *B. cepacia in vitro* (Santos -Ferreira *et al* 1986, Prince 1986), resistance soon develops and the levels of the drug required to be effective in CF respiratory infections would have too many side-effects to be of clinical value. Macrolide antibiotics such as erythromycin , along with lincomycin and its semi-synthetic derivative clindamycin, inhibit protein synthesis by inhibiting dissociation of

peptidyl tRNA from ribosomesLincomycin may be used in conjunction with ceftazidime for *B. cepacia* therapy (Simmonds *et al* 1990)

1.7.4 Drugs inhibiting nucleic acid synthesis

A number of antimicrobial drugs act to inhibit nucleic acid synthesis Their actions can be divided into 3 main groups:

1. **Compounds inhibiting precursor biosynthesis**

These include sulphonamides and trimethoprim. Both drugs rely on the need for bacteria to synthesise folate, whilst mammalian cells take up folate supplied by diet. Sulphonamides are synthetic bacteriostatic agents based upon the structure of azo dyes. Sulphonamides inhibit folic acid biosynthesis as they are structural analogues of p-aminobenzoic acid (PABA). PABA is a substrate for dihydropreroate synthetase (DHPS). Sulphonamides compete with PABA for DHPS and are incorporated by the enzyme into inactive folate analogues with no biological uselfulness. As mammalian cells have no DHPS the sulphonamides have selective toxicity for bacteria.

Trimethoprim belongs to a group of synthetic compounds, 2,4 -diaminopyrimidines that inhibit dihydrofolate reductase (DHFR) an enzyme of folate metabolism. Although most cells, including mammalian cells, have DHFR, trimethoprim is around 50,000 times more active against bacterial DHFR. Trimethoprim is often used in combination with a sulphonamide, sulphamethoxazole, in a combination known as co-trimoxazole. Cotrimoxazole has been used with success in treatment of nosocomial infections of *B. cepacia* and is also initially effective in CF, but quickly develops high levels of resistance (Gilligan 1991). This limits its' use in CF therapy.

2. Compounds that interfere with the DNA template

A wide range of compounds interferes with the DNA template including acridines and phenanthridines such as ethidium that intercalates into the DNA causing disruption. They have poor selective toxicity and would interfere with the patients DNA and RNA replication, along with that of the bacteria.

3. Compounds that inhibit enzymatic action

Inhibitors of RNA polymerase

The rifamycins are a group of closely related antibiotics. Rifampicin is the most widely used. A semi-synthetic antibiotic, it acts by binding to DNA-dependent RNA polymerase and inhibits the initiation of RNA replication. It is selective for bacterial enzymes, having no effect on mammalian RNA polymerases.

Inhibitors of DNA gyrase (topoisomerase II)

The bacterial chromosome consists of DNA as a circular, highly supercoiled molecule, achieved by a number of enzymes. When DNA replication takes place daughter DNA strands may become entangled as a reult of torsional forces involved in supercoiling. Therefore enzymes are required to allow separation of daughter chromosomes and then to catalyse supercoiling of the DNA. The enzymes involved are known as topoisomerases. These enzymes include DNA gyrase, a topoisomerae II enzyme.

Several quinolone derivatives inhibit gyrase activity. The first used clinically was Nalidixic acid synthesised in the 1960s. Several other 4- quinolones have since been

developed notably ciprofloxacin. Quinolones act by binding to DNA gyrase. This causes an impairment of function and the cells produce an 'SOS' response, filamenting and stopping replication of damaged DNA to daughter cells until DNA repair mechanisms can repair damage. The induction of the repair mechanisms whilst there is continued gyrase interference may be fatal to the cell. Mammalian topoisomerase II structure is sufficiently different for quinilones to have a selective reaction for bacterial cells.

Newer fluoroquinolones such as ciprofloxacin are not as well characterised as other classes of antibiotic drugs for use in CF, though significant levels of resistance to ciprofloxacin by *B. cepacia* have been *observed* (Bhakta *et al* 1992). The quinolones have the advantage of being delivered orally and have been employed with some success in prophylaxis of *Ps. aeruginosa* in conjunction with inhaled colistin at the Edinburgh CF centre (Govan & Nelson 1992)

1.7.5 Drugs that inhibit membrane integrity

.Polymixin antibiotics are among the most widely used of these antibiotics. Polymixins are cyclicopolycationic peptides with a fatty acid chain joined by an amide bond. They have bactericidal activity as a result of their interactions with the cytoplasmic membrane causing disorganisation of membrane structure. They are of limited clinical use as they also have an affinity, if somewhat lower, for mammalian membranes. The polymixin colistin has been used in conjunction with ciprofloxacin in the prophylaxis of *Ps. aeruginosa* (Govan and Nelson 1992).

1.7.6 Antibiotic uptake

To be effective antibiotics must be uptaken by the bacterial cell, crossing the cell membrane or membranes to reach their target within the cell or cell wall.

Several classes of antibiotics cross the outer membrane by passive diffusion through porin channels including chloramphenicol, tetracyclines and β-lactams. After entry β-lactams then act upon peptidoglycan synthesis in the cell wall. Chloramphenicol and tetracyclines cross the inner membrane via an active transport system.

Other classes of antibiotics use self-promoted mechanisms to enter the cell. Aminoglycosides cross the cell membranes by the displacement of cations from lipopolysaccharides by the polycationic drug. This then interferes with cross bridging of lipopolysaccharide molecules leading to destabilisation of the membrane and allowing the uptake of the antibiotic. Polymixins are believed to be taken up in a similar way. Quinolones cross membranes both through porin channels and by cationic displacement like aminoglycosides.

1.7.7 Antibiotic therapy in *B. cepacia* infections

The treatment of *B. cepacia* infections has proved very difficult particularly in CF. *B cepacia* is inherently resistant to or quickly develops resistance to many antibiotics (Lewin *et al* 1993). Trimethoprim/sulphamethoxazole (co-trimoxazole) and chloramphenicol have been historically used against *B. cepacia* infections as they demonstrate good activity *in vitro*. β-lactam drugs, particularly antipseudomonal penicillins such as carbenicillin and ticaricillin and cephalosporins have been the main

source of anti-pseudomonal chemotherapy over the last thirty years but have been shown to be largely ineffective against *B. cepacia* (Prince 1986). Aminoglycoside antibiotics, often inhaled by use of a nebulizer have proved effective in CF, particularly against *Ps. aeruginosa* (Friel 1995), sometimes in conjunction with β-lactams. However virtually all *B. cepacia* isolates have inherent resistance to aminoglycosides. Combination therapy with intravenous temocillin and intravenous aminoglycosides has shown some clinical effect (Taylor *et al* 1992) (with 9 out of 12 courses showing positive effect despite resistance to aminoglycosides in most cases), but others have suggested aminoglycoside therapy may predispose for a *B. cepacia* infection (Govan and Nelson 1992). Resistance profiles and minimum inhibitory concentrations (MIC) to a range of the newer and more commonly used antibiotics have been determined for nosocomial isolates (Santos-Ferreira *et al* 1985) and CF isolates (Bhakta *et al* 1992). Both groups demonstrated resistance to aminoglycosides in 90-100% of isolates, whilst resistance to a similar degree to a range of cephalosporins and first generation anti-pseudomonal penicillins such as conbericillin was described in the nosocomial isolates. Activity of newer anti-pseudomonal penicillins was much greater, 67% of nosocomial strains were susceptible to azlocillin and 85% to piperacillin, whilst only 15% of CF isolates were resistant to mezlocillin and only 11% resistant to piperacillin. The third generation aminothiazole cephalosporin, ceftazidime was found to be effective *in vitro*. 93% of nosocomial and around 90% of CF isolates were found to be susceptible to co-trimoxazole (93%) and chloramphenicol (78%). High levels of resistance to quinolones were also found, with 60% of CF isolates being resistant to ciprofloxacin. More recently in the UK, (Lewin *et al* 1993, Butler *et al* 1995) higher levels of resistance

have been described with up to 92% of CF isolates resistant to chloramphenicol, 81% resistant to ceftazidime and 42% resistant to piperacillin.

Studies on experimental antibiotics have demonstrated mixed efficacy. Only 2 of 9 experimental drugs demonstrated activity against *B. cepacia* when tested alongside the currently available antibiotics (Bhakta *et al* 1992). The other 7 antibiotics were a range of cephalosporins and fluorinated quinolones. The cephalosporin cefpirome showed only low level activity with only another deacetylcefotaxime showing a reasonably good level of activity. Studies on fluorinated quinolones have shown increased activity over current drugs such as ciprofloxacin (Espinoza *et al* 1988, Tamura *et al* 1988, Lewin *et al* 1993). Results with cephalosporins have been more mixed (Bosso *et al* 1991, Bhakta *et al* 1992) although cefiprime was shown to have activity by one group (Bosso *et al* 1991), another demonstrated almost complete resistance to it (Bhakta *et al* 1992). The experimental polyene antibiotic faeriefungin demonstrated activity against *B. cepacia* but all *Pseudomonas* species tested were found to be resistant (Mulks *et al* 1990).

Despite their activity *in vitro*, antibiotics used in treatment of *B. cepacia* especially in CF often produce mixed results. Ceftazidime used frequently in treatment is often poor in curing *B. cepacia* CF lung infections (Gold *et al* 1983), as are co-trimoxazole and chloramphenicol (Prince 1986). The antipseudomonal penicllinases are also often ineffective. The use of a β-lactamase inhibitor, clavulanic acid has improved the activity of azlocillin against some strains of *Ps. aeruginosa* (Calderwood *et al* 1982) but does not produce the same effect in *B. cepacia* (Prince 1986). However sulbactam,

another β-lactamase inhibitor, has been found to have its own antibacterial activity against *B. cepacia* (Jacoby and Sutton 1989). The reasons for the poor *in vivo* activity are not clear. It may be due in part by failure to obtain a sufficiently high concentration of antibiotic within the lung to be effective (Gold *et al* 1983) whilst *in vitro* studies have suggested the CO_2 levels and pH in the CF lung may decrease the susceptibility of *B. cepacia* but not *Ps aeruginosa* (Corkhill *et al* 1994).

The use of carbapenems, potent broad-spectrum β-lactam antibiotics, shows some promise. Meropenem appears to show better activity *in vitro* than several commonly used antibiotics to a range of CF and environmental strains (Lewin *et al* 1993, Butler *et al* 1995) whilst imipenem appears to have a synergistic effect in combination with a range of antibiotics (Kumar *et al* 1989). Although *B. cepacia* produces a carbapenem hydrolysing β-lactamase (Baxter and Lambert 1994) it is thought to be inactivated by β-lactamase inhibitors such as tazobactam and clavulanic acid and it appears the carbapenem or imipenem in conjunction with inhibitors may be successful (Simpson *et al* 1993). Environmental strains of *B. cepacia* often display high levels of resistance to many antibiotics. However recent research suggests they are somewhat more susceptible to drugs such as ceftazidime, piperacillin and meropenem (Butler *et al* 1995, Pitt *et al* 1996). It is of particular concern that 2 isolates from CF patients in Scotland and Wales were found to be resistant to all antibiotics tested against them including ceftazidime, piperacillin, ciprofloxacin and meropenem (Lewin *et al* 1993). Such multi-resistant isolates present a particular problem in management of CF, and their potential for predominance is of great worry.

1.8 Antibiotic resistance in *B. cepacia*

1.8.1 Mechanisms of resistance

Since the initial years of antibiotic therapy it has been known that certain species of bacteria were not killed by the actions of antibiotics. It was therefore recognised that antibiotic-resistant bacteria existed (Russell and Chopra 1990).

Two broad-based categories of antibiotic resistance are recognised; intrinsic and acquired or extrinsic resistance. Intrinsic resistance involves the inherent features of the cell being responsible for the failure of antibiotic action. Intrinsic resistance is normally expressed from chromosomal genes. It is the basis of much of the resistance of *B. cepacia*, *Ps. aeruginosa* and many opportunistic bacteria.

Acquired resistance is usually the result of selective pressure placed upon bacteria by antibiotic therapy. It is usually acquired either by mutation of chromosomal genes or by the acquisition of plasmid or transposon genes. The emergence and spread of acquired resistance is of great concern to medical science as resistance genes are relatively easily acquired or passed on and can result in multiple resistance. This causes difficulty in treatment of resistant bacterial infections.

Bacteria have developed a number of strategies of antibiotic resistance (Russell and Chopra 1990). The main mechanisms include:

1. Alteration or inactivation of antibiotic.

2. Antibiotic target site in bacterial cell is insensitive to the antibiotic and can still perform its normal function

3. Decreased accumulation of the antibiotic either by impaired uptake of the antibiotic or by increased efflux.

4. By -passing the antibiotic sensitive step by expression of an alternative target site that is insensitive to antibiotic action.

5. Overproduction of the target site so much higher concentrations of antibiotic are required to be effective.

6. Absence of an enzyme or metabolic pathway that is the target site for the antibiotic.

1.8.2 Intrinsic and acquired resistance in *B. cepacia*

Resistance in *B. cepacia* is primarily intrinsic,based largely around impermeability of the cell to antibiotics (Wilkinson and Pitt 1995b), though there is some evidence of acquired mechanisms of resistance, there is little knowledge of antibiotic resistance encoded by plasmids.

Trimethoprim resistant dihydrofolate reductase (DHFR) has been found to be produced in resistant *B. cepacia* CF isolates (Burns *et al* 1989b). The resistant DHFR producing isolates had MIC's of > 1000 $\mu g\ ml^{-1}$ compared with ,maximum levels of 10 $\mu g\ ml^{-1}$ in non-producers, though only 39% were susceptible..This suggested though DHFR is the main mechanism of resistance in some strains, others have different resistance mechanisms.

Resistance to β-lactams

Chromosomally encoded β-lactamases in *B. cepacia* have best known since the 1970s. *B. cepacia* 249 is able to utilise penicillin G as a carbon source (Beckman and Lessie 1979), whilst β-lactamase deficient forms were not. In 1980 an inducible β-lactamase was obtained from *B. cepacia* (Hirai *et al* 1980). The majority of β-lactamases described in *B. cepacia* are inducible penicillinases (Baxter and Lambert 1994). One of the best described of these is the product of the *penA* gene in *B. cepacia* 249 (Prince *et al* 1988, Proneca *et al* 1993). This was found to have homology with *amp-C* encoded β-lactamase from *Enterobacteriacae* and *Ps. aeruginosa*. The sequence of the *penA* gene is more closely related to the *Enterobacteriacae* suggesting it may have been acquired by *B. cepacia* from the *Enterobacteriocae*. There is evidence that all *B. cepacia* strains also express carbapenem hydrolysing β-lactamases or carbapenemases (Simpson *et al* 1993). Bacteria very rarely produce carbapenemases although *B. cepacia* along with *Stenotrophomonas maltophilia* and some strains of *Serratia*, *Aeromonas* and *Bacteroides* appear to be the exception. A carbapenem hydrolysing inducible β-lactamase has been isolated from a clinical isolate of *B. cepacia* (Baxter and Lambert 1994). This was found to hydrolyse carbapenems, penicillins and cephalosporins including ceftazidime. The action of the enzyme was inhibited by metal ion chelators such as EDTA but this action was reversed by zinc indicating the requirement for metal ions- a metallo beta lactamase. The enzyme was named PCM-1 (*Pseudomonas cepacia* metallo enzyme 1) and was subsequently found to be present in 8 other *B. cepacia* clinical isolates (Baxter and Lambert 1994).

Intrinsic antibiotic resistance is very important in *B. cepacia*. The resistance is primarily based upon the impermeability of the membrane to antibiotics Three main mechanisms of intrinsic resistance have been found

1. Decreased or altered membrane porin proteins
2. The nature of lipopolysaccharide in the outer membrane prevents binding of cationic antibiotics
3. An active antibiotic efflux pump decreases accumulation of drugs

1. Resistance by diminished porin content

The outer membrane of Gram negative bacteria has a low permeability to hydrophillic molecules. Porins are outer membrane proteins that form transmembrane pores that allow diffusion of hydrophillic molecules into the bacterial cell. *B. cepacia*, like *Ps aeruginosa*, is highly impermeable to hydrophillic molecules such as hydrophillic antibiotics including β-lactams. The permeability is around ten times less than for *E. coli* (Parr *et al* 1987). Studies indicated that the porins of *B. cepacia* and *Ps. aeruginosa* have a small pore size thereby allowing only little diffusion of hydrophillic antibiotics, leading to general low susceptibility to such antibiotics. Porins in *B. cepacia* have been found to consist of an 81 kDa protein complex formed from two units of 36 kDa thought to be the main porin protein and 27 kDa protein. Reduced amounts of the 36 kDa protein and reduced expression of the 27 kDa protein have been found in CF antibiotic resistant isolates and β-lactam resistant mutants (Aronoff 1988). These alterations caused reduced permeability leading to resistance to ciprofloxacin and ceftazidime. Thediminished expression of the 27 kDa protein lead to speculation of its

role as the porin protein or as a major component of the porin complex. Salicylate induced resistance in a susceptible *B. cepacia* isolate has also been demonstrated to be the result of diminished expression of porin proteins (Burns and Clark 1992). The presence of a 40 kDa outer membrane protein was suppressed by growth with salicylate. The absence of the protein leads to decreased permeability to chloramphenicol, trimethoprim and ciprofloxacin but not to ceftazidime. The 40 kDa protein was termed OpcS (salicylate-suppressible outer membrane protein), whilst the 36 kDa protein was found not to decrease with the presence of salicylate was termed OpcP.

2. Outer membrane LPS Structure

The outer membrane of *B. cepacia* and its' lipopolysaccharide structure prevent polycationic antibiotics such as polymixins and aminoglycosides binding to the outer membrane (Wilkinson and Pitt 1995b). These antibiotics have a self promoted uptake mechanism that requires cross bridging with LPS to cause cationic displacement to allow them to permeate through the bacterial membrane. Unlike *Ps. aeruginosa*, *B. cepacia* cells will not bind the fluorescent probe dansyl-polymixin (Moore and Hancock 1986). It was thought that cation-binding sites on LPS were in some way concealed or protected. More recent studies (Cox and Wilkinson 1991, Wilkinson and Pitt 1995b) also suggested poor binding to the outer membrane. This finding was based upon the LPS structure of *B. cepacia* having few phosphate or carboxylate groups and the presence of the protonated amido sugar 4-amino-4 deoxyarabinose (Ara 4N). This results in a weakly anionic structure that therefore has poor binding to the cationic antibiotics.

3. Antibiotic Efflux Pump

Decreased permeability to chloramphenicol, trimethoprim and ciprofloxacin in *B. cepacia* had been reported on the basis of a 21.9 kb DNA fragment in *B. cepacia* (Burns *et al* 1989a). Analysis of a subcloned 3.4 kb fragment identified one complete, and one partial open reading frame (ORF) (Burns *et al* 1996b). Based upon sequence data, outer membrane protein analysis, protein expression systems and lipoprotein assay, homology was found with 2 of 3 components of the *Ps aeruginosa mexA-mexB-oprM* antibiotic efflux operon (Nikaido 1994). A lipoprotein with homology to *oprM* was found with the complete reading frame, homology at protein level to the C terminus of the *mex*B protein product was found with the partial reading frame. This analysis suggests the presence of an efflux pump, that would actively 'pump out' antibiotics thereby preventing accumulation within the cell. Such an efflux pump would be similar to the proposed *mexA-, mexB-oprM* pump proposed in *Ps. aeruginosa*.

1.9 Plasmids in *B. cepacia*

Plasmids are DNA molecules that can replicate independentlyof , and co-exist with the host chromosome(s) (Day 1982). They are in essence mini chromosomes usually formed of negatively supercoiled circles of double stranded DNA. Plasmids carry genes that encode for a wide variety of functions including antibiotic resistance, heavy metal resistance, bacterial conjugation, bacteriocin production, antibiotic production and metabolism of simple and complex compounds (Summers 1996). Plasmid genes do not normally encode for essential functions in the bacterial host or plasmid under normal conditions, but rather encode for properties that allow growth under abnormal growth conditions; extending host range into atypical environments or encoding for increased metabolic diversity (Day 1982, Summers 1996). Plasmid genes are transferable between individual bacteria and between species and therefore are important agents in horizontal gene transfer and bacterial evolution. Plasmids are most frequently transferred by bacterial conjugation, but may also be transferred via bacteriophage (transduction) or by uptake of naked DNA (transformation) (Amabile-Cuevas and Chicarel 1992).

Relatively few *B. cepacia* plasmids have been studied in detail, with few phenotypic characteristics assigned to plasmid genes.. Gonzalez and Vidaver (1979) found plasmids in 15 of 16 environmental and clinical isolates. The plasmids found varied in size from 9 to 120 MDa (14-185 kb). The type strain, ATCC 25416 contained a 185 kb plasmid. The presence of such large plasmids suggested a role in antibiotic resistance to the researchers. Subsequently smaller plasmids encoding antibiotic resistance were found (Williams *et al* 1979, Hirai *et al* 1982). Lennon and DeCicco

(1991) found 84 % of clinical, pharmaceutical and environmental isolates (31/37) harboured one to four plasmids between 2.7 to 222 kb in size. Large plasmids of 146 to 222 kb were commonly found in clinical isolates with a higher antibiotic resistance phenotype so, like Gonzalez and Vidaver, Lennon and DeCicco suggested these large plasmids played a role in antibiotic resistance without assigning any phenotypic traits to specific plasmids. Large plasmids of 208 and 212 kb were also found in strains obtained from onions. Restriction digest analysis showed significant differences between 212 kb plasmids in pharmaceutical and environmental strains but demonstrated identity between two 222 kb plasmids in pharmaceutical isolates. Analysis of the 222 kb plasmids showed high levels of homology, suggesting a close relationship or common source of these plasmids. This group sized the type strain cryptic plasmid at 212 kb whilst Rodley *et al* (1995) sized it at 200 kb as compared to the 185 kb described by Gonzalez and Vidaver (1979). In addition transfer of plasmid DNA into *B. cepacia* by conjugation was demonstrated. R751, a plasmid of incompatibility Inc P-1, encoding for trimethoprim resistance was transferred from *Ps. aeruginosa* into four *B. cepacia* isolates at a transfer frequency of between 4×10^{-7} to 7×10^{-4} donor cells ml^{-1} of mating mix. Transfer of plasmid from a *B. cepacia* R751 transconjugant to three other *B. cepacia* recipients at frequencies of 2×10^{-6} to 3×10^{-5} donor cells ml^{-1} of mating mix was achieved. This showed the ability of plasmid DNA, and significantly antibiotic resistance, to be conjugatively transferred into and between *B. cepacia* strains. Despite the high levels of *B. cepacia* plasmids in these studies McKevitt and Woods (1984) found plasmids in only 11 of 48 (23%) *B. cepacia* isolates from CF patients as compared to 84% of plasmid containing isolates found by Lennon and DeCicco (1991) in non CF isolates. Seven isolates contained a 60 MDa (92 kb)

plasmid, another isolate contained 2 plasmids of 100 mDa (154 kb) and 50 mDa (77 kb) and the final isolate a 50 MDa (77 kb) plasmid. No phenotypic characteristics were assigned to the plasmids.

More recently a number of conjugative plasmids encoding catabolic phenotypes have been described (Bhat *et al* 1994, Haak *et al* 1995, Shields *et al* 1995, Prakash *et al* 1996). These are discussed in detail later.

Insertion elements have been found within *B. cepacia* plasmids. Byrne and Lessie (1994) described two IS 3 family insertion elements , IS 401 and IS 408, within the 170 kb cryptic plasmid pTGL 1 in *B. cepacia* ATCC 17616. These elements have been demonstrated to play a role in the genetic rearrangement of the plasmid. The novel insertion elements IS 1071 and IS 1471 have also been found in *B. cepacia* plasmids (Xia *et al* 1996). IS 1471 inserts into IS 1071 found on the 102 kb plasmid pIJB 1. These sequences do not belong to any known IS group.

1.9.1 Antibiotic resistance plasmids in *B. cepacia*

The role of plasmids encoding for antibiotic resistance in *B. cepacia* has not been extensively explored (Wilkinson and Pitt 1995). Prior to the study of Lennon and DeCicco (1991), which did not assign any resistance phenotypes, there had been only two descriptions of antibiotic resistance plasmids in *B. cepacia*. Transformation of plasmid DNA from *B. cepacia* 4G9 into *E. coli* HB101 resulted in the discovery of a 2.9 MDa (4.4 kb) plasmid, pJW 2, encoding tetracycline resistance and a 5.2 MDa (8 kb) plasmid , pJW 3 , encoding for constitutive ampicillin resistance (Williams *et al*

1979). Electron microscopy also permitted visualisation and identification of a 1.78 MDa plasmid , pJW 1. These plasmids were of insufficient size to be conjugative.

A larger 32 MDa (49 kb) plasmid , Rms 425, was found to mediate streptomycin and mercury resistance in *B. cepacia* GN11131 (Hirai *et al* 1982). Transformation of plasmid DNA was achieved between GN11131 a non-resistant mutant , ML5062, and *Ps. aeruginosa* PAO 21242 (Hirai *et al* 1982). Plasmid DNA was obtained by equilibrium gradient ultracentrifugation. It was also possible to transform the plasmid from *Ps. aeruginosa* PAO 21242 transformants to *E. coli, B. cepacia* and other *Ps. aeruginosa* strains. Mating experiments were also conducted from *Ps. aeruginosa* and *E. coli* plasmid-transformants into *E. coli* and *Ps. aeruginosa* nalidixic acid resistant recipients to determine conjugative properties of the plasmid. Transfer was achieved at a low frequency (2×10^{-7} to $< 10^{-8}$) by membrane filter mating, but no transfer was found by broth mating. No transfer was achieved using *B. cepacia* as the donor , possibly due to a defect in the transfer mechanisms. Incompatibility testing placed the plasmid in the Inc P-1 group. The resistance to streptomycin was believed to be due to a streptomycin phosphotransferase (Hirai *et al* 1982).

Subsequently there has been little published evidence of antibiotic resistance plasmids in *B. cepacia*, though a number of broad host range mercury resistant plasmids have been described(Rochelle *et al* 1988, Sabate *et al* 1994). The 78 kb plasmid pQM3 was found in a river epilithon *B. cepacia* strain (Rochelle *et al* 1988) and the 54 kb plasmid pAMJ6 (Sabate *et al* 1994) from natural water. pAMJ6 was placed in incompatibility group Inc P-1.

1.9.2 Degradative plasmids in *B. cepacia*

The majority of the plasmids described as encoding catabolic pathways in *B. cepacia* are large conjugative plasmids (Bhat *et al* 1994, Haak *et al* 1995, Shields *et al* 1995). Shields and co-workers found a 108 kb TOM plasmid encoding for the unique toluene-*ortho*-monooxygenase (TOM) toluene degradative pathway. This pathway is significantly different from the pathways of the common TOL plasmids in having an initial monooxygenase step, then proceeding by toluene hydroxylation. TOL plasmids encode the ability to oxidise toluene via a benzoate intermediate. On the basis of the Tom enzyme the plasmid was named TOM. Curing of the plasmid removed the ability to grow on toluene or phenol. Conjugative transfer of TOM to other *B. cepacia* isolates, *E. coli* and into a TOM cured isolate was achieved . However transfer to *E. coli* was not accompanied by toluene degradation, possibly due to lack of recognition of the TOM promoter, though cloning of *tom*A and *tom*B genes from the *tom* operon and transfer into a recombinant *E. coli* lead to expression of the genes. The recombinant *E. coli* did not encode for the full pathway, but showed trichloroethylene degradation activity, supporting the suggestion that the promoter was not recognised in the *E. coli* transconjugants.

Haak *et al* (1995) described a 70 kb plasmid, pBAH 1, encoding for a 2-halobenzoate 1,2 dioxygenase in *B. cepacia* 2 CBS. Conjugative transfer of the plasmid was investigated by first transforming into *E.* coli and then using the transformant as a donor for conjugal transfer into a *Ps. putida* recipient. The gene cluster encoding for the enzyme, *cbdABC*, was sequenced and cloned. Cloned genes were subsequently

expressed in *Ps. putida*, which could then metabolise chlorobenzoates. Sequence homology to an *Acinetobacter sp.* 1,2 dioxygenase was found.

Bhat *et al* (1994) described a 90 kb plasmid, pMAB 1, encoding genes involved in 2,4-D degradation in *B. cepacia* CSV 90. The role of the plasmid was deduced by curing and transformation studies. Replica plating on media with and without 2,4-D was used to find spontaneously cured CSV 90 clones unable to grow on 2,4-D. These 2,4-D^- isolates were subsequently checked for presence of the plasmid. The cured strains were found to have lost pMAB1. Transformation of cured isolates with pMAB1 was conducted using electroporation. The electroporated cells were able to grow on 2,4-D and contained a 90 kb plasmid. Cloning of a 10 kb plasmid fragment and expression in *E. coli* found a gene, *tftC*, encoding for a dichlorocatechol dioxygenase. Sequence homology analysis found the gene to be identical to a gene from a 2,4,-D degrading *Alcaligenes eutrophus* plasmid, pJP4. However restriction mapping of the pJP4 and pMAB1 plasmids found them to be different overall, implying that the gene had been acquired from the other plasmid or a common source.

In contrast small plasmids, e.g. a 5 kb *p*-nitrophenol degradative plasmid, are also found in *B. cepacia* (Prakash *et al* 1996). This degradative property could be transferred conjugally, either through plasmid mobilisation or through incorporation into a transposon. The plasmid also encoded for zinc resistance.

1.10. Comparative properties and relationships of clinical and environmental *B. cepacia* isolates

The potential to acquire *B. cepacia* infection from environmental sources is of serious concern, especially in view of its use as an agent of biocontrol in agriculture and bioremediation. There are considerable gaps in the knowledge about transmissibility of *B. cepacia,* although there is strong evidence for person to person transmission , there are large contrasts in the transmission rates in CF centres world-wide (Govan *et al* 1996) and between CF siblings (Cazzola *et al* 1996). It is therefore important to understand relationships between clinical and environmental isolates of *B. cepacia.*

Early studies compared clinical and environmental isolates on the basis of bacteriocin production, plasmid content, pectinolytic activity and the ability to macerate onion tissue (Gonzalez and Vidaver 1979). The results divided isolates into two broad groups. Generally environmental isolates had strong pectinolytic and onion macerating activity, whilst clinical isolates had much weaker activity. Both groups contained plasmids and were found to be equally lethal to mice in *in vivo* studies. More recent phenotypic studies between rhizosphere and clinical strains again showed differences in properties (Bevivino *et al* 1991). The rhizosphere strains were able to grow at a wider range of temperatures and were antagonistic to a wider range of phytopathogenic fungi than clinical strains. Clinical strains could bind to human uroepithelial cells, whereas environmental strains could not. In contrast to this Butler *et al* (1995), found CF

strains, notably the epidemic ET12 strain, were as capable of causing *in vitro* onion maceration as environmental strains.

The study of Yohalem and Lorbeer (1994) used virulence to onion, catabolic ability, pigment production, gelatin hydrolysing and pectinolytic activity to group 218 clinical, soil and phytopathogenic isolates into four phenoms or clusters . Phenom I was a heterogeneous group of onion macerating and non-macerating strains. Phenom II were highly pectinolytic, onion macerating bacteria many of which produced diffusible pigments. This group included the species type strain. Phenom III were non-macerating and contained the majority of clinical isolates. Phenom IV contained only strains isolated from celery fields. The use of DNA-DNA and DNA-rRNA hybridisation studies have shown *B. cepacia* to consist of at least four genomovars (Vandamme 1995, Govan *et al* 1996). Most environmental isolates fall into genomovar I with the species type strain. The strains associated with a rapid decline in CF all fall into genomovar III.

Differences in antibiotic resistance between clinical and environmental isolates have also been noted. Although the majority of isolates are resistant to many antibiotics intrinsically, differences in resistance to quinolones and newer B-lactam drugs have been found; clinical isolates having higher resistance levels to piperacillin, ceftazidime, meropenem and ciprofloxacin (Butler *et al* 1995).

1.10.1 Epidemiology of environmental and clinical strains.

Comparative analysis of clinical and environmental strains has suggested that environmental strains are incapable of causing human infection (Bevivino *et al* 1994). This is almost certainly not the case as 'swamp foot' lesions in US marines have been demonstrated to be caused by *B. cepacia* (Taplin *et al* 1971). Evidence of any epidemiological relationships between clinical and environmental strains is poor, hampered by a lack of information. Investigations by clinicians of the prevalence of *B. cepacia* in the environment have suggested that it is difficult to isolate *B. cepacia* from the environment using media routinely used in clinical laboratories. *B. cepacia* was recovered from 18 % of samples taken in the homes of CF patients infected with *B. cepacia*, and from 4 % of samples from the general urban environment (Mortensen *et al* 1995). A previous study had found *B. cepacia* at lower rates(Fisher *et al* 1993). *B. cepacia* was only recovered from 1 % of samples taken in patient homes and 4.5 % of samples in food bars and supermarket salad and vegetable counters (Fisher *et al* 1993). The main source of *B. cepacia* appears to be organic soils, where an isolation rate of over 20 % has been found (Butler *et al* 1995).

The development of effective typing techniques has enabled better epidemiological studies of environmental strains. Fisher *et al* (1993) used ribotyping to demonstrate that the predominant CF ribotype could also be found in the environment, where it could be a potential reservoir of infection. In the UK no evidence of potential environmental transmission has been found suggesting the environment is of little hazard to CF patients. Butler *et al* (1995) used PFGE macrorestriction analysis with the endonucleases *Xba* I and *Spe* I and produced unique profiles for each of 12 tested

environmental strains. These differed from 11 clinical isolates including the epidemic ET12 strain. No environmental strain tested has yet been placed in genomovar III with the ET 12 CF epidemic strain.

Typing of N. American isolates by multilocus enzyme electrophoresis and ribotyping has placed all tested environmental strains into separate electropherotype clusters to CF or nosocomial *B. cepacia* isolates (Johnson *et al* 1994). However a close genetic relationship between the ET 12 epidemic strain and the species type strain has been found. More recent evidence from Italy (Amalfitano *et al* 1996, Cazzola *et al* 1996), has suggested that person-to-person transmission and environment-to-person transmission may occur in CF patients.

The knowledge regarding the infectious potential of environmental *B. cepacia* strains is very limited, particularly as so few isolates have been studied. This has lead to caution being advised over the use of *B. cepacia* as an agent of biocontrol and bioremediation by some groups (Butler *et al* 1995, Govan *et al* 1996).

1.11. Aims of study

The primary aim of the study is to characterise and compare *B. cepacia* isolates obtained from the sputum of CF patients and a range of environmental sources. The majority of the CF isolates were from the South Wales area, so to assess the relationships between environmental and clinical isolates in this area a number of *B. cepacia* strains were isolated from a range of environmental sources. These isolates, along with a number of others from clinical and environmental sources from the UK and world-wide, were then be characterised phenotypically by their phytopathogenicity and antibiotic resistance profiles. Genetic characterisation would be made on the basis of PCR ribotyping and macrorestriction analysis. The replicon arrangement of the genome was investigated by PFGE.

The second major aim of the study was to investigate plasmids in *B. cepacia.* Plasmid content and size was characterised by a range of plasmid DNA isolation methods. Identification and characterisation of antibiotic resistance plasmids was undertaken by curing studies and mating experiments. Conjugal transfer of antibiotic resistance and other plasmids was investigated by laboratory mating experiments. Transfer of plasmids from environmental sources was also investigated.

In addition to these main aims a number of other factors were investigated: assessment and development of selective media for isolation of *B. cepacia* from the environment, development of quantitative tests for phytopathology of onion, assessment of methods for plasmid DNA isolation in *B. cepacia* and the application of molecular typing methods to environmental *B. cepacia* isolates.

Chapter 2 Isolation and identification of *Burkholderia cepacia*

2.1 Introduction

As described in the general introduction, a number of media have been developed for the isolation of *B. cepacia* from clinical specimens, most notably CF sputum, and the environment (Wu and Thompson 1984,Gilligan *et al* 1985, Hagedorn *et al* 1987). *B. cepacia* from CF patients in the UK is primarily isolated from liquefied sputum on Mast cepacia (PC) agar based on that described by Gilligan *et al* (1985) (Govan and Nelson 1992). Confirmatory identification is usually based on the API 20NE system and/or biochemical reactions.

Environmental surveys in the UK have used Malka minimal media supplemented with 300U ml^{-1} of polymixin for initial enrichment prior to growth on the Mast medium (Pitt and Govan 1993, Butler *et al* 1995). For surveillance of surfaces, swabs moistened in the supplemented Malka medium have been used to swab the surface, these were either plated directly onto the Mast cepacia agar or placed in the Malka media and grown for 5 days at 30°c prior to plating. Soils and vegetation (around a teaspoonful) were placed directly into the Malka medium and grown for 5 days prior to plating on the Mast medium. Waters were filtered through a membrane filter; the filter then being treated as soil. Similar methods have been employed in the USA (Fisher *et al* 1993) The low levels of *B. cepacia* isolated from the environment in other studies (Fisher *et al* 1993, Butler *et al* 1995, Mortensen *et al* 1995), suggest that more efficient media that allow the growth of *B. cepacia* but inhibit the growth of other species may be developed.

2.2 Materials and methods

Materials

i. Chemicals

Sterile liquid paraffin (BDH Ltd., Poole, Dorset)

Salicin , Analar grade (BDH Ltd.,)

Maltose, Analar grade (BDH Ltd.,)

Lactose, Analar grade (BDH Ltd.,)

Industrial methylated spirits (BDH Ltd.)

Trypan blue (Gurr, BDH Ltd.)

Crystal violet (Gurr, BDH Ltd.)

Tetracycline (Sigma Chemical Co., Poole, Dorset)

Gentamicin (Sigma Chemical Co,)

Tetramethyl-*p*-phenylenediamine dihydrochloride crystals (Sigma Chemical Co.)

ii. Strains

Strains obtained from PHLS, Dublin and Type Strain Collections

Strainsobtained from the PHLS are shown in Table 2.1, those obtained from Dublin in Table 2.2 and those obtained from the National Industrial and Marine and National Plant Pathogenic Collections in Table 2.3. Isolates, with sources of other species used in the study are shown in Table 2.4

Maintenance and storage of strains

As problems have been reported with maintenance of *B. cepacia* at 4° C (Gilardi 1983), all isolates obtained were frozen in broth culture with 20 % v/v sterile glycerol

as a cryoprotective agent at –70°C. Isolates were recovered from frozen stocks as and when required.

Table 2.1 ***B. cepacia*** **isolates from PHLS Cardiff**

ISOLATE	SOURCE	OTHER INFORMATION
C1 (KW)	Cardiff CF Clinic	Paediatric Isolate
C5 (JR)	Cardiff CF Clinic	Paediatric Isolate
C11 (DR)	Cardiff CF Clinic	Paediatric Isolate, Sibling of C5 (JR)
C23 (RW)	Cardiff CF Clinic	Paediatric Isolate, Sibling of C1 (KW)
C49 (NL)	Cardiff CF Clinic	Paediatric Isolate
C51 (AM)	Cardiff CF Clinic	Paediatric Isolate
C59 (BH)	Cardiff CF Clinic	Adult Isolate
C79 (KT)	Cardiff CF Clinic	Adult Isolate
C81 (CP)	Cardiff CF Clinic	Adult Isolate
C93 (AW)		
C95 (PS)	Cardiff CF Clinic	Adult Isolate
C96 (HW)	Cardiff CF Clinic	Adult Isolate
C116 (JJ)	Cardiff CF Clinic	Paediatric Isolate (Swansea)
C187 (KP)	Cardiff CF Clinic	Adult Isolate
C190	Quality Control Isolate	Sent as part of Quality Control study
C205 (JS)	Cardiff CF Clinic	Adult Isolate
A562	Scotland (Edinburgh) CF Isolate	
A548	Scotland (Edinburgh) CF Isolate	
C1858	Scotland (Edinburgh) CF Isolate	
C1860	Scotland (Edinburgh) CF Isolate	
J478	Strasbourg (France) CF Isolate	
J543	Strasbourg (France) CF Isolate	
J2552	Rhizosphere of *Carludoucas palmata*	Isolated in tropical aquatic house

Table 2.2 ***B. cepacia*** **isolates from Dublin**

ISOLATE	SOURCE	OTHER INFORMATION
D1 (LB)	Dublin CF clinic	Paediatric Isolate (Failed to Grow)
D2 (DC)	Dublin CF clinic	Paediatric Isolate
D3 (MF)	Dublin CF clinic	Paediatric Isolate
D4 (SH)	Dublin CF clinic	Paediatric Isolate
D5 (SR)	Dublin CF clinic	Paediatric Isolate
D6 (MR)	Dublin CF clinic	Paediatric Isolate

Table 2.3 **NCIMB and NCPPB** ***B. cepacia*** **strains**

STRAIN	SOURCE	OTHER INFORMATION
NCIMB 9085	Forest soil, Trinidad	Synonymous with ATCC 17759 NCTC 10661
NCIMB 9087	Rotting tree trunk, Trinidad	Synonymous with ATCC 17761
NCIMB 9088	Jamaica	Synonymous with ATCC 17769 Produces purple phenazine pigment
NCIMB 9092	Jamaica	Synonymous with ATCC 17772 Produces purple phenazine pigment
NCPPB 2993	Onion, U.S.A	Synonymous with ATCC 25416 Species Type Strain
NCPPB 3480	Onion Papua New Guinea	

Strains obtained from National Collection of Industrial and Marine Bacteria (NCIMB) (Aberdeen, Scotland), and National Collection of Plant Pathogenic Bacteria (NCPPB) (Harpenden, Hertfordshire).

Table 2.3 Other (Non-*B. cepacia*) Strains used in this study

STRAIN	SOURCE	OTHER INFORMATION
Burkholderia gladioli NCPPB 2478	Onion, India	*allicola* pathovar
Escherichia coli NCTC 10418		
Pseudomonas aeruginosa PAO1/pQM1/ R300B	University of Wales College Cardiff (UWCC)	pQM1= 251kb mercury resistance plasmid
Ps. aeruginosa PAO1/R300B	UWCC	R300B = 8.5kb plasmid
Ps. aeruginosa PAO 2002 /RP1	UWCC	RP1 = 65kb plasmid Encodes for β-lactamase
E. coli V517	PHLS, Colindale	Size marker: contains 8 plasmids
E. coli 39R	PHLS, Colindale	Size marker: contains 4 plasmids
Alcaligenes eutrophus NCIMB 11842	Soil	Species Type Strain
Stenotrophomonas maltophilia 5018	PHLS, Cardiff	
S. maltophilia 5851	PHLS, Cardiff	
S .maltophilia 4543	PHLS, Cardiff	

Other strains used from the collection of Microbiology Section, School of Biomedical Sciences, University of Wales Institute Cardiff

Culture of *B. cepacia* strains

For overnight culture on agar plates *B. cepacia* strains were grown on Nutrient agar plates for 24 h at 35°C from single colonies sub-cutlured from other plates maintained at room temperature,or from broth culture from recovered isolates store frozen at –70°C.

Overnight broth cultures were grown in static culture from single colonies on agar plates in 10 ml of Nutrient broth at 35°C for 16h to give a culture of approximately 10^{7} cfu ml^{-1} unless otherwise stated

The above conditions apply to all overnight cultures throughout the thesis, unless indicated otherwise.

iii. Media

Nutrient agar plates (Oxoid, Unipath, Basingstoke, Hants.)

Nutrient broth (Oxoid, Unipath)

Pseudomonas cepacia (PC) media and selectatabs (Mast Diagnostics, Bootle, Merseyside)

Andrade peptone water (Oxoid, Unipath)

MacConkey agar plates (Oxoid, Unipath)

Bacteriological agar (Agar No.2) (Oxoid, Unipath)

TB-T agar (Hagedorn *et al* 1987) and variations as noted in the method section.

Onion (*Allium cepa)*, sound and apparently disease-free, purchased from local supermarkets.

iv. Solutions and commercial kits

API 20NE kits, James, NIT , NIT 2 and Zn reagents (BioMerieux, Marcy l'Etoile, France).

MacFarland Standards (BioMerieux)

v. Equipment

Glass Petri dish and plate spreader

3 ml Bijou bottles

Durham tubes

vi. Disposable consumables

90 mm Petri dishes

Sterile toothpicks

Sterile polypropelene 20 ml Universal bottles

Filter paper (Whatman No 1, Whatman International Ltd., Maidstone, Kent)

Sterile stockingette (or muslin)

Methods

i. Environmental sampling

Two separate rounds of environmental sampling were conducted, each at a range of representative sites at industrialised and rural areas throughout South Wales. For the first round of sampling (Survey #1) samples were plated only on to Mast PC agar. For the second set of samples (Survey #2) each was plated on PC, TB-T, TB-T-CN, Onion-T and Onion T-CN media.

Soils:

Around 2-3g of soil was collected into sterile universal containers. This was mixed with 10 ml of sterile water by vortexing for 5 minutes. This was allowed to settle before preparing dilutions of the upper aqueous layer in sterile water from neat to 10^{-2}. 100µl of each dilution was spread onto the surface of selective agar plates.

Rhizosphere (Root Shake):

Plant roots were removed gently from the ground and placed into a sterile container. 10ml of sterile water was added to the container. The samples were vortexed for 5 minutes. The sample was allowed to settle and the upper layer treated as for soil samples.

Water / sediment

Around 10ml of water and sediment were aseptically collected into sterile universal containers in an upstream direction. Samples were vortexed for 5 minutes and allowed to settle. 100µl of the upper layer was spread onto the surface of PC plates.

Rotten Onions:

A sterile loop was used to scrape the scales of rotten onions. This was then streaked onto the surface of PC plates.

With all types of sample the plates were incubated at 30°C for 48 h. Any growth was recorded and sub-cultured onto Nutrient agar plates for identification.

ii. Identification/characterisation

A. Identification by API 20NE system

Prior to identification using the API 20NE system, all isolates were Gram stained and their morphology investigated. Gram negative rods were then identified by API 20NE. Other isolates were discarded.

The API 20NE system is a standardised system using 20 tubes and cupules on a strip that contains dehydrated reagents and media that form 8 conventional biochemical and 12 assimilation tests. An oxidation test is employed as the 21st test. The oxidase test

used was based on that of Kovacs (Barrow and Feltham 1993). A fresh solution of medium was prepared by adding 10 ml of sterile distilled water to a few crystals of tetramethyl-*p*-phenyldiamine dihydrochloride. A few drops of this solution were used to moisten filter paper in a sterile petri dish. The isolate was tested by picking a colony from a fresh plate culture with a toothpick and smearing onto the filter paper. A positive reaction leads to the development of a dark purple colour in 30 seconds. *Ps. aeruginosa* was used as a positive control, *E. coli* as a negative control. The results of the oxidase test were recorded on the API 20NE report form.

For the remainder of the tests, the manufacturer's protocol was followed. After 24 h the results were recorded and added together to give a numerical profile. The numerical profile was entered into the API LAB v.3.1 program on a personal computer or compared with the Analytical Profile Index to identify the isolate. In some cases identification is not possible after 24hours due to slow growth . For such results the TRP and NO_3 tests were covered with paraffin to prevent any ongoing reactions interfering with the other tests. The strip was re-incubated for another 24 hours and read again. The profiles were then calculated and the identification process repeated .

B. Confirmatory biochemical tests for *B. cepacia*

In a number of cases API 20NE results gave doubtful or unacceptable profiles for *B. cepacia*, with *Ps. fluorescens, Ps. putida, Ps. paucimobilis, Stenotrophomonas maltophilia* and *Aeromonas* species given as alternatives. To determine the identity, acidification of sugars in ammonium salt media was chosen from Cowan and Steel's Manual for Identification of Medical Bacteria (Barrow and Feltham 1993). *B. cepacia*

produces acid from maltose, lactose and salicin whilst *Ps. fluorescens* or *Ps .aeruginosa* will not give acidification with lactose or maltose, *Ps. putida* or *Stenotrophomonas maltophilia* will not produce acid with salicin. *Ps. paucimobilis* will not grow on MacConkey agar whilst *B. cepacia* will grow.

Acidification of sugars

Andrade peptone water was made up to the manufacturer's instructions and 1.8 ml placed into Bijou bottles. Inverted Durham tubes were placed into the bottles, which were then sterilised by autoclaving. 10 % w/v stock sugar solutions were produced in distilled water (2 g in 20 ml of water) and filter sterilised. 200 µl of the stock sugar solution was added to each Bijou aseptically to give a final sugar concentration of 1 %.

The bottles were inoculated with the isolates to be tested. The inoculated bottles were incubated for 14 days at 37 °C. The bottles were checked daily for acidification. Acidification is indicated by presence of a pink- red colour. Presence of bubbles in the Durham tube indicates gas production.

Growth on MacConkey agar.

Isolates were streaked onto the MacConkey agar and then incubated at 37° for 48 hours. After incubation plates were checked for growth.

Development of selective media and assessment of media for *B. cepacia* isolation

On the basis of apparent selectivity of agar containing onion homogenate for *B. cepacia* during phytopathogenicity testing (see Chapter 3), it was decided to use onion within a selective medium for *B. cepacia* as this may allow select ion by supporting

growth of *B. cepacia* whilst inhibiting growth of other species. Onion homogenate was produced by adding equal volumes of onion tissue, including skin, to equal volumes of sterile saline mixing in a Kenwood kitchen blender for 2 min and then filtering through conical flask along with the onion homogenate and 1µl ml^{-1} of trace element solution to give a 2% agar with 20% onion homogenate. The agar was autoclaved at 121°C, 15 p.s.i. for 15 minutes and then maintained at 50°C in a waterbath. The agar was either poured directly to give Onion (O) Agar, or tetracycline added at 20µg ml^{-1} ,the same level as in TB-T, to give Onion-T (O-T) agar, gentamicin at 5µg ml^{-1} to give Onion-CN (O-CN) agar or both tetracycline and gentamicin to give Onion-T-CN (O-T-CN) agar.

TB-T agar was produced as described by Hagedorn *et al* (1987), using crystal violet as the antifungal component. TB-T was also supplemented with gentamicin at 5µg $ml^{-1}$to give TB-T-CN agar. Mast selective agar (PC) was produced according to manufacturer's instructions.

The suitability of each medium to select for *B. cepacia* and the optimum temperature for isolation was tested using 26 *B. cepacia* isolates and 12 isolates from other species, including isolates reported as growing on *B. cepacia* selective media (Gilligan *et al* 1985 , Hagedorn *et al* 1987). Three sets of plates were produced to test growth at 20°C, 30°C and 37°C. Nutrient agar plates were included as a control for growth of isolates.

1 ml of overnight growth of isolates was pipetted into the PTFE dish of the Mast Multipoint Inoculator. The sterile pins of the inoculator were placed into the holder

and the inoculator was operated to introduce spots of culture onto the surface of 3 sets of Nutrient agar, PC, TB-T, TB-T-CN, O, O-T, O-CN and O-T-CN plates. One set of plates was incubated at 20°c for 48 h, one at 30°C for 48 h and the final set was incubated at 37°C for 48 h. After the incubation growth on each plate was recorded On the basis of their selectivity, the ability to support growth of *B. cepacia* but to inhibit other species, the more successful media were chosen for use in the secon round of environmetal sampling.

2.3 Results

2.3.1 Environmental survey #1

The sites from which isolates were obtained, along with Ordnance Survey references, and nature of samples from which they were obtained are shown in Table 2.5 below.

Table2.5 Strains isolated in environmental survey #1; Source, sample type and media used for isolation

Isolate	Sample Site (Ordnance Survey Ref.)	Sample Type	Media Used For Isolation
PW 1	R. Rhondda Tributary, Cwmparc, Treorchy, Rhondda Cynon Taff (SS 945 952)	Water	PC
PW 2	R. Rhondda Tributary, Cwmparc, Treorchy, Rhondda Cynon Taff (SS 944 952)	Water	PC
PW 3	R. Ogwr, Ogmore Castle, Bridgend (SS 883 760)	Water	PC
PW 4	R. Ely, Coed Ely, Rhondda Cynon Taff (ST 022 857)	Water	PC
PW 5	Woodland, Coed Ely, Rhondda Cynon Taff (ST 022 857)	Soil	PC
PW 6	R. Rhondda Tributary, Cwmparc, Treorchy, Rhondda Cynon Taff (SS 945 953)	Water	PC
PW 7	Woodland, Coed Ely, Rhondda Cynon Taff (ST 022 858)	Soil	PC
PW 8	-	Rotten Onion	PC

2.3.2 Growth of *B. cepacia* and other species on selective media.

Growth of the 26 clinical and environmental *B. cepacia* isolates and the 12 strains from non- *B. cepacia* species are shown in Table 2.6 for growth at 20°C, Table2.7 for growth at 30°C and Table 2.8 for growth at 37°C.

Key for tables 2.6 ,2.7 & 2.8

++	Strong growth of isolate
+	Weak growth of isolate
-	Growth not supported

Table 2. 6 **Growth of *B. cepacia* and other strains on selective media at 20°C**

Isolate	NA	PC	TB-T	TBT-CN	O	O-T	OCN	OCN-T
Clinical *B. cepacia* isolates								
C1	++	-	-	-	-	-	-	-
C5	++	-	-	-	-	-	-	-
C11	++	-	-	-	-	-	-	-
C23	++	-	-	-	-	-	-	-
C49	++	-	-	-	-	-	-	-
C51	+	-	-	-	-	-	-	-
C79	+	-	-	-	-	-	-	-
C95	+	-	-	-	-	-	-	-
C116	+	-	-	-	-	-	-	-
C187	+	-	-	-	-	-	-	-
C190	+	-	-	-	-	-	-	-
C205	+	-	-	-	-	-	-	-
A548	+	+	+	+	+	+	+	+
A562	+	+	+	+	+	+	+	+
D2	+	-	-	-	-	-	-	-
D4	+	+	+	+	+	+	+	+
D5	+	-	+	+	+	+	+	+
Environmental *B. cepacia* isolates								
NCPPB 2993	+	+	-	+	+	+	+	+
NCIMB 9085	+	-	-	-	-	-	-	-
NCIMB 9092	-	-	-	-	-	-	-	-
J2552	+	+	-	-	+	-	+	-
PW1	-	-	-	-	-	-	-	-
PW2	+	+	-	-	+	-	+	-
PW3	+	+	-	-	+	-	+	-
PW4	+	+	-	-	+	-	+	-
PW5	+	+	-	-	-	+	+	+
Non-*B. cepacia strains*								
Ps. aeruginosa PA01/ pQM1/R300B	+	+	+	-	+	-	+	-
Ps. aeruginosa PA01	+	-	+	-	+	-	+	-
Ps. aeruginosa PaW1	+	-	-	-	+	-	+	-
Aeromonas hydrophilia	+	-	-	-	-	-	-	-
Serratia marcescens	+	-	+	-	+	+	+	+
Ps. putida UWC1	+	+	-	-	+	-	+	-
Micrococcus luteus	+	-	-	-	+	-	+	-
Bacillus cereus	+	-	-	-	-	-	-	-
Bacillus subtilis	+	-	-	-	-	-	-	-
Bacillus mycoides	+	+	+	+	+	+	-	-
E. coli NCTC 10418	+	-	-	-	+	-	+	-
E. coli V517	+	-	-	-	+	-	+	-

Table 2. 7 Growth of *B. cepacia* and other strains on selective media at 30°C

Isolate	NA	PC	TB-T	TBT-CN	O	O-T	OCN	OCN-T
Clinical *B. cepacia* isolates								
C1	+	-	-	-	+	-	-	-
C5	+	-	-	-	+	-	+	-
C11	+	-	-	-	+	-	+	-
C23	+	-	-	-	+	-	+	-
C49	+	-	-	-	+	-	+	+
C51	+	-	-	-	+	-	-	-
C79	+	-	-	-	+	-	+	-
C95	+	-	-	-	+	-	-	-
C116	+	-	-	-	-	-	-	-
C187	+	-	-	-	-	-	-	-
C190	+	-	-	-	-	-	-	-
C205	+	-	-	-	-	-	-	-
A548	+	+	+	+	+	+	+	+
A562	+	+	+	+	+	+	+	+
D2	+	-	-	-	-	-	-	-
D4	+	+	+	+	+	+	+	+
D5	+	+	+	+	+	+	+	+
Environmental *B. cepacia* isolates								
NCPPB 2993	+	+	+	+	+	+	+	+
NCIMB 9085	+	-	-	-	-	-	-	-
NCIMB 9092	+	-	-	-	-	-	-	-
J2552	+	+	-	-	+	-	+	-
PW1	+	-	-	-	-	-	-	-
PW2	+	+	-	-	+	-	+	-
PW3	+	+	-	-	+	-	+	-
PW4	+	+	-	-	+	-	+	-
PW5	+	+	-	-	+	-	+	-
Non-*B. cepacia strains*								
Ps. aeruginosa PA01/ pQM1/R300B	+	-	+	-	+	+	+	-
Ps. aeruginosa PA01	+	-	+	-	+	+	+	-
Ps. aeruginosa PaW1	+	-	-	-	+	-	+	-
Aeromonas hydrophilia	+	-	-	-	-	-	-	-
Serratia marcescens	+	-	+	-	+	+	+	+
Ps. putida UWC1	+	+	-	-	+	-	+	-
Micrococcus luteus	+	-	-	-	+	-	+	-
Bacillus cereus	+	-	-	-	-	-	-	-
Bacillus subtilis	+	-	-	-	-	-	-	-
Bacillus mycoides	+	+	+	+	+	+	-	-
E. coli NCTC 10418	+	-	-	-	+	-	+	-
E. coli V517	+	-	-	-	+	-		

Table 2.8 Growth of *B. cepacia* and other strains on selective media at 37°C

Isolate	NA	PC	TB-T	TBT-CN	O	O-T	OCN	OCN-T
Clinical *B. cepacia* isolates								
C1	+	-	-	-	-	-	-	-
C5	+	-	-	-	-	-	-	-
C11	+	-	-	-	-	-	-	-
C23	+	-	-	-	+	-	-	-
C49	+	-	-	-	-	-	-	-
C51	+	-	-	-	+	-	-	-
C79	+	-	-	-	+	-	-	-
C95	+	-	-	-	-	-	-	-
C116	+	-	-	-	-	-	-	-
C187	+	-	-	-	-	-	-	-
C190	+	+	-	-	+	-	-	-
C205	+	-	-	-	-	-	-	-
A548	+	-	+	+	+	+	+	+
A562	+	+	+	+	+	+	+	+
D2	+	-	-	-	-	-	-	-
D4	+	+	+	+	+	+	+	-
D5	+	-	+	+	+	+	+	-
Environmental *B. cepacia* isolates								
NCPPB 2993	+	+	+	+	+	+	+	+
NCIMB 9085	-	-	-	-	-	-	-	-
NCIMB 9095	-	-	-	-	-	-	-	-
J2552	+	+	-	-	+	+	+	+
PW1	-	-	-	-	-	-	-	-
PW2	+	+	-	-	+	-	+	-
PW3	+	-	-	-	-	-	-	-
PW4	+	+	-	-	+	-	+	-
PW5	+	-	-	-	+	-	-	-
Non-*B. cepacia strains*								
Ps. aeruginosa PA01/ pQM1/R300B	+	-	+	-	+	-	+	-
Ps. aeruginosa PA01	+	-	+	-	+	-	+	-
Ps. aeruginosa PaW1	+	-	-	-	+	-	+	-
Aeromonas hydrophilia	+	-	-	-	-	-	-	-
Serratia marcescens	+	+	+	+	+	+	+	+
Ps.putida UWC1	+	+	-	-	-	-	-	-
Micrococcus luteus	+	-	-	-	-	-	-	-
Bacillus cereus	+	-	-	-	-	-	-	-
Bacillus subtilis	+	-	-	-	-	-	-	-
Bacillus mycoides	+	+	+	+	+	+	-	-
E. coli NCTC10418	+	-	-	-	+	-	+	-
E. coli V517	+	-	-	-	+	-		

The percentage of *B. cepacia* and non- *B. cepacia* isolates grown on each media at each incubation temperature are shown in Tables 2.9,.2.10 and 2.11 below:

Table 2.9 **Percentages of *B. cepacia* strains able to grow on selective media at 20°C**

	NA	PC	TB-T	TBT-CN	O	OT	OCN	OT-CN
% *B. cepacia* Isolates Grown	92	37	17	21	42	21	42	21
% Other Species Grown	100	17	33	8	67	17	59	17

Table 2.10 **Percentages of *B. cepacia* strains able to grow on selective media at 30°C**

	NA	PC	TB-T	TBT-CN	O	OT	OCN	OT-CN
% *B. cepacia* Isolates Grown	100	37	19	19	69	19	58	19
% Other Species Grown	100	17	33	8	67	17	59	17

Table 2.11 **Percentages of *B. cepacia* strains able to grow on selective media at 37°C**

	NA	PC	TB-T	TBT-CN	O	OT	OCN	OT-CN
% *B. cepacia* Isolates Grown	88	27	19	19	50	23	31	15
% Other Species Grown	100	25	33	17	50	17	42	17

2.3.3 Environmental survey #2

13 *B. cepacia* isolates were obtained from 73 samples collected from sites in the South Wales area. 6 were isolated on Mast PC medium, 3 each on TB-T medium and Onion-T medium and 1 in TB-T-CN medium. Only 2 isolates PW17 and PW21 were isolated by different media from the same sample, though their profiles by API 20NE differed .

Isolates were identified as *B. cepacia* by API 20NE with confirmation by additional biochemical test when neccessary. A number of other Gram negative species were found to grow on the selective media. These were identified as *Ps. fluorescens, Ps .paucimobilis, Xanthomonas* (*Stenotrophomonas*)species) and *Flavobacterium* sp. on Mast PC media in a few cases, *Pseudomonas, Aeromonas* and *Flavobacterium* sp. were identified plates of each of the other media, particularly TB-T. Gram positive cocci were also found on a number of plates but no further identification was undertaken. Heavy fungal growth was also noted on a number of plates, particularly Onion -T plates.

The site, including Ordnance Survey reference, and sample type, along with the media on which isolates were obtained is shown in Table 2.12 below.

Table 2.12 Strains isolated in environmental survey #2 : Source, sample type & media used for isolation

Isolate	Sample Site (Ordnance Survey Ref.)	Sample Type	Media Used For Isolation
PW 9	Ystradfellte, Powys (SN 932 138)	Soil	PC
PW 10	Storey Arms, Powys (SN 981 204)	Water	TB-T
PW 11	Llandaff, Cardiff (ST 157 782)	Soil	Onion-T
PW 12	Llwyn-On Reservoir, Powys (SO 008 125)	Water	PC
PW 13	Storey Arms, Powys (SN 981 204)	Rhizosphere (Root Shake)	PC
PW 14	R. Taff, Llandaff, Cardiff (ST 163 783)	Water	Onion-T
PW 15	Cantref Reservoir, Powys (SN 995 158)	Rhizosphere (Root Shake)	TB-T
PW 16	Ystradfellte, Powys (SN 932 138)	Water	PC
PW 17	Cantref Reservoir, Powys (SN 995 158)	Water	Onion-T
PW 18	Llwyn-On Reservoir, Powys (SO 008 125)	Rhizosphere (Root Shake)	PC
PW 19	Blaen Lilia, Powys (SN 929 159)	Rhizosphere (Root Shake)	TB-T
PW 20	Storey Arms, Powys (SN 981 205)	Water	PC
PW 21	Cantref Reservoir, Powys (SN 995 158)	Water	TB-T-CN

2.3.4 Recovery rates of *B. cepacia* in environmental Surveys #1 & #2

The recovery rates of *B. cepacia* from environmental samples are given as the percentage of samples tested on each media from which *B. cepacia* was isolated. The percentage recovery rate for each of the selective media is shown in Table 2.13 below:

Table2.13 % Recovery Rate of *B. cepacia* in Environmental Surveys for Selective Media

Media	Numbers Isolated /Samples Tested	Recovery Rate %
Mast PC Agar	14/106	13
TB-T Agar	3/73	4
TB-T-CN Agar	1/73	1.5
Onion-T Agar	3/73	4
Onion-T-CN Agar	0/73	0

In total 21 isolates were recovered from 106 samples giving a total recovery rate of 20%.

2.4 Discussion

2.4.1 Assessment of selective media

There are two major factors associated with selective media for *B. cepacia.* The first of these is the ability or failure of *B. cepacia* to grow on the media, the second the ability of the media to inhibit the growth of other species. The most selective media in terms of inhibiting growth of other species were TB-T supplemented with gentamicin and onion agar with tetracycline and tetracycline and gentamicin, the commercial Mast PC media and TB-T. Whilst onion agar and onion-CN allowed the growth of most *B .cepacia* isolates but also supported growth of many other species.

With the PC agar at 20°C and 30°C only 17% and at 37°C 25% of other the species tested grew(Tables 2.6, 2.7). The isolates that grew were *Ps. putida*, *Serratia marcescens* and *Bacillus mycoides* . Growth of both *Ps. putida* and *Serratia marcescens* on PC medium have been previously described (Gilligan *et al* 1985, Mast Diagnostics 1994). The relatively low level of growth of *B .cepacia* was somewhat surprising. Gilligan *et al* (1985) reported that 76% of *B. cepacia* stock isolates grew at 35°C , whilst only 35% of isolates grew at 20°C and 30°C, even less at 37°C, in this study.

The TB-T medium supplemented with 5μg ml^{-1} of gentamicin allowed growth of only 8% of non- *B. cepacia* isolates(*Bacillus mycoides*) at 20°C and 30°C, *Serratia* also grew at 37 °C. TB-T without the addition of gentamicin also supported the growth of 2 *Ps .aeruginosa* isolates giving a total of 33% of non- *B. cepacia* isolates that grew on the medium. As with PC medium, *Serratia* species have been reported to grow on TB-

T medium (Hagedorn *et al* 1987). The addition of gentamicin though apparently increasing selectivity, appeared not to affect the growth of *B. cepacia*, with both media allowing the growth of 19%)of *B. cepacia* isolates. The levels of growth were again rather lower than expected.

The onion agar supplemented with tetracycline and tetracycline and gentamicin allowed the growth of 2 non-cepacia isolates, *E. coli* and *Serratia marcescens*, a total of 17 % of the non- *B. cepacia* isolates. Both Onion-T and Onion-T-CN grew 19% at 20°C and 30°C, though Onion-T-CN had a decreased yield of 15% at 37°C but Onion-T had an increased yield of 23%. These levels being similar to those for TB-T.

Onion agar with no antibiotic supplementation was the most successful medium for growing *B. cepacia* with a 69% culture success rate at 30°C. Onion supplemented with gentamicin supported growth of 58% of isolates at 30°C. However these high levels of *B. cepacia* growth were also accompanied by growth of other species. Onion agar supported the growth of upto three-quarters of the non-*B. cepacia* species, The agar supplemented with gentamicin supported growth of up to 59% of other species tested.

A selective medium for use in environmental sampling would ideally allow growth of the bacterium being selected, whilst inhibiting the growth of other species. Although onion agar, either with or without gentamicin allowed growth of more *B. cepacia* strains, their inability to inhibit other species would limit their usefulness in sampling studies as the growth of so many other species would greatly increase the workload of identification and may overgrow any *B. cepacia* strains. TB-T or TB-T supplemented with gentamicin or Onion agar with tetracycline or tetracycline and gentamicin were

reasonably good inhibitors of other species, particularly where gentamicin is included. However the growth of *B. cepacia* on these media was relatively poor, less than 20% of isolates growing. Mast PC agar also showed good species selectivity, but allowed growth of higher numbers of *B. cepacia,* up to 35%. On the basis of this, PC agar would appear to be the best medium for environmental surveys. However nearly two-thirds of *B. cepacia* isolates tested failed to grow on this medium. Therefore it was decided to use a range of media in the subsequent environmental survey in order to increase recovery rate of *B. cepacia.* Mast PC medium, TB-T, TB-T-CN, O-T and O-T-CN agars were used for the subsequent environmental survey, Survey #2 , on the basis of their specificity.

Temperature appeared to cause some variance in the growth of *B. cepacia.* Overall 30°C seemed to be the optimum temperature with the highest or equal highest amounts of *B. cepacia* growth on all but O-T-CN agar. In addition growth of other species was inhibited better at this level, notably of *Serratia marcescens* which did not grow on PC or TB-T-CN at 30°C but grew at 37°C. On the basis of this 30°C was chosen as the optimum growth temperature for environmnetal sampling..

The development of the Onion agar as a selective media was moderately successful. The use of onion alone or with gentamicin was not suitable as a selective medium as it failed to inhibit the growth of other species. Onion with tetracycline or tetracycline and gentamicin was at least as successful in this study as TB-T agar with similar levels of inhibition of other species and growth of *B. cepacia.* However it was not as effective as the commercial medium

2.4.2 Recovery rate of *B. cepacia* from the environment

On the basis of recovery rates from the environment it would appear that Mast PC agar was the most successful medium for isolation of *B. cepacia* from the environment. A recovery rate of 13% was obtained using PC, compared to 4% for TB-T and Onion-T and less than 2% for Onion -T-CN. In Survey #2 the total recovery rate for all media was 18%.

The earlier Survey #1 had a higher recovery rate of 24%, 8 *B. cepacia* isolates from 33 samples and sites, using PC agar alone. Combining the results of both surveys, 21 *B. cepacia* strains were isolated from 106 samples and sites, giving a total recovery rate of 20%. This compares well to the 21% recovery rate described by Butler *et al* (1995). The use of PC agar alone would have given a total recovery rate of 13% (14/106). The other media isolated a total of 7 *B. cepacia* strains from 73 samples, a total recovery rate of 10%. Although the individual recovery rates for both TB-T and O-T were low at 4%, in combination with PC medium they increased the total recovery rate from 13% for PC alone, to 20% for all media. This represents a substantial increase, suggesting that the use of multiple media in sampling may be effective than PC alone.

It is notable that a number of strains were isolated from relatively polluted areas. Isolates PW1, PW2 and PW6 were obtained from a river tributary downstream of old mineworkings and a slag heap. PW4, PW5 and PW7 were isolated downstream of the site of a former coal mine and coking works, a site where considerable remedial work to make safe areas of toxic contamination were being undertaken at the time of sampling. The versatile nutritive and degradative properties of *B. cepacia* could allow it to thrive in such contaminated ecological niches .

Butler *et al* (1995) surveyed sites in botanical gardens, with the majority of *B. cepacia* strains recovered , 10 of 12, were isolated in soil or rhizosphere samples from tropical, tropical aquatic, orchid and cycad or rhododendron houses. Such sites would be warm and moist with rich soils, ideal for growth of soil bacteria. A 'tropical' environment , albeit artificial, would be of relatively limited relevance to any link between the environmental strains of *B. cepacia* and those found in CF patients. CF is most prevalent in Caucasian populations in Northern Europe and North America, where a more temperate climate occurs (Tsui 1992). The levels of *B. cepacia* found in this study of soil and water samples from industrial, urban and rural sites in South Wales is arguably more representative of the environment to which CF patients are exposed than the samples analysed by Butler and co-workers (1995).

The overall recovery rates are considerably higher than those of around 4% found by Fisher *et al* (1993) and Mortensen *et al* (1995). These studies challenged the notion of *B. cepacia* as an ubiquitous organism in the environment (Holmes 1985), due to the low levels of recovery. However, these surveys were restricted to such areas as foodstores, salad bars and the 'built' environment. As pointed out by Butler *et al* (1995), the sample sites did not include soils, natural waters or rhizosphere samples that form the main environmental reservoirs of *B. cepacia.* The recovery rates in this study would appear to support this.

A notable point is that in all the sites surveyed, only one sample gave *B. cepacia* on more than one media. The isolates obtained from this site at Cantref Reservoir were PW 17 on onion-T agar and PW 21 on TB-T-CN agar. The two isolates showed different API profiles, PW17 producing a β-glucosidase hydrolysing esculin, and a

protease hydrolysing gelatine and β-galactosidase whilst PW21 does not. PW17 also shows considerable *in vitro* phytopathogenicity to onion, whilst PW21 does not (Chapter 3, p128)). Unfortunately the failure of PW21 to grow from frozen stock did not allow typing by PCR ribotyping of macrorestriction analysis to confirm PW21 and PW17 as different strains. (Chapter 8, p.250). The use of these techniques showed differences amongst all environmental isolates in this study.

2.4.3 Efficacy of selective media in environmental and clinical isolation of *B. cepacia*

From the assessment of the selective media it would appear that none would be truly suitable for an environmental survey. The media most selective for *B. cepacia*, Mast PC agar, grew only 35 % of *B. cepacia* strains tested, though did inhibit most other species tested. In addition several of the clinical *B. cepacia* isolates tested failed to grow on the Mast PC media despite being originally isolated on this media. The isolates tested were obtained from frozen stocks, so it would appear that either storage at -70 °C has affected the ability of these isolates to grow on the PC media, or more worryingly that the PC medium does not recover *B. cepacia* as efficiently as previously thought. A second problem, that of other species growing on selective media has been noted by clinicians and laboratory workers. This may lead to mis-identification, particularly of *Stenotrophomonas maltophilia* as *B. cepacia* (Burdge *et al* 1995). This problem has been addressed to some extent by the development of *Burkholderia cepacia* selective agar (BCSA), a medium with greater selectivity for *B. cepacia* (Henry *et al* 1997).

Hagedorn *et al* (1987) commented that media developed for isolation of clinical *B. cepacia* strains from sputum, or media developed for isolation of *B. cepacia* from

waters or pharmaceutical solutions (Wu and Thompson 1984), use antibiotics as their selective component. It was suggested this would limit their suitability for soil sampling, as many organisms resistant to these antibiotics are found in soils, in particular fungi, and could overgrow the plates. In addition the high levels of antibiotics in BCSA would suggest that it is not suitable for environmental isolation of *B. cepacia*, particularly as environmental isolates appear to be less resistant to antibiotics than clinical isolates and such isolates would fail to grow on such media due to the high levels of antibiotics (Butler *et al* 1995). In contrast to the views of Hagedorn and co-workers, the Mast PC medium , developed primarily for isolation from sputum, was more effective in the environmental surveys than TB-T developed for isolation from soils. PC had a recovery rate of 8 to 24% compared to only 4% for TB-T.

The Onion agar supplemented with tetracycline showed similar levels of selectivity to TB-T both in the assessment of the media and in the environmental survey. In Survey #2 TB-T and O-T both isolated 3 *B. cepacia* strains from 73 samples, a 4% recovery rate. The rate for the onion medium may have been higher if heavy fungal growth on plates had not prevented further analysis of a number of bacterial colonies. This fungal growth could be eliminated by the addition of an anti-fungal component such as the crystal violet or nyaststin used in TB-T , or by cycloheximide which inhibits synthesis of the 80S ribosome protein synthesis (I.J. Bruce Personal communication).

Overall, considering the results of both the surveys and testing of selective media, it would appear that the Mast PC agar is the most effective single medium tested for isolation of *B. cepacia*. However the results of Survey #2 would indicate that the use

of PC in combination with other media such as TB-T and O-T could be considerably more effective in environmental surveys. This is particularly true if one considers that in 73 samples in Survey #2, only one sample yielded *B. cepacia* on more than one media, even in this case the isolates showed different phenotypic properties. In effect this means for all the isolates detected, the other 4 media used failed to isolate the same *B. cepacia* strain. This suggests a 'scatter gun' approach in which a variety of media is more effective than a single media in isolating *B. cepacia* from environmental sources.

2.4.4 Isolation and identification of *B. cepacia*: General considerations

The overall recovery rate of 20 % was similar to that of previous studies for soil and rhizosphere samples. A possible *caveat* to these results is that only 35% of *B. cepacia* isolates grew on PC agar in the assessment tests of media suitability. If such a rate were transferred to environmental surveys then around two-thirds of tested samples may contain *B. cepacia* strains not detected by the media. If this were to be the case then *B. cepacia* may be far more prevalent in the environment that previously thought. Such levels, potentially up to 50 or 60% , may have implications in the role of the environment as reservoir of *B. cepacia* .

Despite uncertainties about the levels of *B. cepacia* in the environment, the environmental surveys succeeded in providing 21 *B. cepacia* strains for further analysis, along with those clinical and environmental strains obtained from sources such as the PHLS.

Chapter 3 Phytopathogenicity of *Burkholderia cepacia strains*

3.1 Introduction

Burkholder first described *Burkholderia cepacia* as a phytopathogen, identifying it as the cause of 'sour skin' onion rot (Burkholder 1950). The ability of *B. cepacia* to act as phytopathogen *in vitro* rotting onions through the maceration onion tissue by enzymes leading to soft and slimy tissue has been known for some time, and has been utilised as a test of onion pathogenicity (Lelliott and Stead 1987). The ability to cause rot or maceration *in vitro* is a useful test in characterisation of *B. cepacia*, and recently onion maceration has been used as one of the criteria for typing *B. cepacia* in phenoms or genomovars (Yohalem and Lorbeer 1994, Vandamme 1995, Govan *et al* 1996).

3.2 Materials and methods

Materials

i. Chemicals

70 %V/V alcohol (Industrial Methylated Spirits) (BDH Ltd, Poole, Dorset)

ii. Strains

Overnight 10 ml broth cultures (Chapter2 p.98)of

B. cepacia strains (As described in Chapter 2)

B. gladioli pv *allicola* NCPPB 2478 (As described in Chapter 2)

Ps. aeruginosa PA01 & PA2002 (As described in Chapter 2)

iii. Media

Nutrient broth (Oxoid CM1, Unipath, Basingstoke, Hants.)

Bacteriological agar (Agar No1) (Oxoid L11, Unipath)

Sterile saline (0.85% w/v)

iv. Solutions and other materials

Onions (*Allium cepa)*. Sound and apparently disease-free, purchased from local supermarkets.

'Sterile' onions (autoclaved in 'honey jars' at 15 p.s.i . 121°C for 15 mins)

v. Equipment

Domestic kitchen blender (Kenwood)

No. 4 (8mm) cork borer

vi. Disposable consumables

Sterile stockingette (or muslin)

Sterile toothpicks

Sterile polythene bags (laboratory stomacher bags)

90 mm petri Dishes

Methods

i. *In vitro* maceration of onion slices (host test for phytopathology

The method used was based on that of Lelliott and Stead (1987). Onions were wiped with alcohol and then cut aseptically into slices of approximately 5mm; skins were not removed. Tissue slices were places into sterile petri dishes and two shallow nicks of around 2mm were made into the onion surface. 200µl of overnight culture of *B. cepacia strains* were used to inoculate the surface of the onion slices. The inoculated onion tissue was incubated at 30^0 C for 72 hours. After incubation levels of maceration were recorded using a sterile needle as a probe.

Overnight cultures of *Ps. aeruginosa* PAO 1 and PAO 2002/ RP1 and sterile saline were used to inoculate control tissue.

Phytopathogenic *B. cepacia strains* should cause a slimy, yellowish rot of inner scales giving rise to soft macerated tissue (Bradbury 1986, Lelliott and Stead 1987). *B. gladioli* also causes maceration but can be distinguished as it gives rise to a 'glassy' appearance and does not produce the distinct 'sour' smell associated with *B. cepacia.*

Onions were also tested in the same manner to assess their suitability in quantitative assays or in isolation media. Onions were placed in jars and autoclaved at 15 p.s.i., 121^0 C for 15 min.

The various degrees of macerationobserved in the onion slices. were scored into 5 levels of phytopathology:

4+	yellow/brown discoloration. Strong 'sour' odour.
3+	Maceration evident in majority of onion tissue. Yellow or yellow/brown discoloration through most of tissue. Strong 'sour' odour.
2+	Patches of maceration and discoloration. 'Sour' odour.
1+	Little evidence of maceration. Slight discoloration. Slight 'sour' odour.
0	No maceration, discoloration or odour.

ii Whole onion pathogenicity (Lelliott and Stead 1987)

Alcohol wiped onions were inoculated into the their outer scales with sterile toothpicks dipped into a heavy overnight *B .cepacia* culture. The inoculated onions were placed in sterile stomacher bags. The bags were sealed and incubated in the dark at room temperature. The onions were checked for indications of rot at 3 , 4 , 7 , 10 , 14 , 21

and 28 days. At 28 days the onions were sliced in half to check for signs of pathology, such as discoloration or softening of the inner scales.

iii. Quantitative assay of onion maceration

200 g of onion tissue without roots wiped with alcohol prior to use was placed in the goblet of a Kenwood kitchen blender. The blender goblet was surface sterilised with alcohol prior to use. 200 ml of sterile saline was added to the tissue, which was then homogenised for 90 s. The onion homogenate was filtered through two layers of sterile stockingette into a sterile container. The onion homogenate was added to molten bacteriological agar at concentrations of 0, 1, 2, 5, 10, 25, and 50% v/v. The agar was prepared to a final concentration of 2% after addition of the homogenate, sterilised by autoclaving at 15 p.s.i. at 121°C for 15 mins. Molten agar was maintained at 45°C in a waterbath prior to pouring.

The onion agar was poured into petri dishes and allowed to set and dry. Wells were cut into the agar plates using a No.4 (8mm) Cork borer. 150 µl of overnight culture of *B. cepacia* along with *Ps. aeruginosa* and sterile saline controls were added to the wells. The plates were then incubated at 30° C for 72 h. Alternatively 10 µl of overnight culture were spotted onto the surface of uncut plates which were then incubated as above. After incubation any clear zones, where onion was macerated, in the plates was recorded.

3.3 Results

3.3.1 *In vitro* maceration of onion tissue

The results obtained for the *in vitro* maceration of onion slices are shown in Table 3.1.

Maceration of a slice of onion tissue by *B. cepacia* NCPPB 2993 is shown in Plate 3.1.

Plate 3.1 *In vitro* maceration of onion tissue by *B. cepacia* NCPPB 2993: incubation at 30 °C for 72h

Table 3.1 ***In vitro*** **maceration of onion tissue slices (host test) by** ***B. cepacia***

Level of Maceration	Strain	Source of strain
4+	NCPPB 2993	Rotten onion
3+	PW17	Water
	PW2	Water
	PW6	Water
	A562	Clinical ,Edinburgh CF patient
	NCPPB 3480	Rotten onion
	PW11	Soil
	B. gladioli NCPPB 2478*	Rotten onion
2+	PW20*	Water
	PW19	Rhizosphere Soil
	NCIMB 9085	Soil
	NCIMB 9087	Rotting Tree Trunk
	A548	Clinical ,Edinburgh CF patient
1+	J543	Clinical, Strasbourg CF patient
	C93	Clinical, Cardiff CF patient
	PW8	Rotten Onion
	PW14	Water
	PW4	Water
	PW1	Water
	C79	Clinical, Cardiff CF patient
	C23	Clinical, Cardiff CF patient
	C190	Clinical, Cardiff CF patient
	C116	Clinical, Cardiff CF patient
	C5	Clinical, Cardiff CF patient
	C1	Clinical, Cardiff CF patient
	NCIMB 9092	Not known
	C1858	Clinical ,Edinburgh CF patient
	J478	Clinical, Strasbourg CF patient
	J2552	Soil
	D6	Clinical, Dublin CF patient
0	PW3	Water
	PW5	Soil
	PW7	Soil
	PW9	Soil
	PW10	Water
	PW13	Rhizosphere Soil
	PW15	Rhizosphere Soil
	PW18	Rhizosphere Soil
	PW21	Water
	PW22	Water
	PW12	Water
	C1860	Clinical ,Edinburgh CF patient
	D2	Clinical, Dublin CF patient
	D3	Clinical, Dublin CF patient
	C59	Clinical, Cardiff CF patient

Table 3. 1 (continued)

Level of maceration	Strain	Source of strain
0	C51	Clinical, Cardiff CF patient
	C11	Clinical, Cardiff CF patient
	D2	Clinical, Dublin CF patient
	D3	Clinical, Dublin CF patient
	C95	Clinical, Cardiff CF patient
	C96	Clinical, Cardiff CF patient
	C205	Clinical, Cardiff CF patient
	C187	Clinical, Cardiff CF patient
	Sterile Saline	Control
	Ps. aeruginosa PAO2002 RP1	Control
	Ps. aeruginosa PAO 1	Control

Note: Strains marked with an asterix (*) NCPPB 2478 and PW20 produced a different form of rot without discoloration and the distinct smell, but with a 'glassy' appearance.

Maceration of sterile onion slices

Autoclaving of onion has little effect . The ability of *B. cepacia* to macerate onion in *vitro* can be measured using autoclaved onion tissue, though obviously the process softens the onion tissue somewhat prior to addition of the test isolate. .The results obtained are shown in Table 3.2:

Table 3.2 ***In vitro*** **maceration of sterile (autoclaved) onion slices**

Strain	Level of Rot	Comments
B. cepacia NCPPB 2993	4+	Appearance very similar to that of 'normal' onion inoculated with isolate
B. cepacia NCPPB 3480	3+	Appearance very similar to that of 'normal' onion inoculated with isolate
B. cepacia C79	1+	Some increased softness, but otherwise as 'normal' onion inoculated with the isolate.
Ps. aeruginosa PAO2002 /RP1	0	No rot observed, but slightly softer than normal onion
Sterile Saline	0	No rot observed, but slightly softer than normal onion

***In vitro* maceration of whole onion**

The results obtained are shown in Table 3.3 below

Table 3.3 ***In vitro* maceration of whole onions (host test) by *B. cepacia* strains**

Incubation time	Strain	Observations
72 h	PW 8	No rot observed
	NCPPB 2993	No rot observed
	C79	No rot observed
	Sterile Saline	No rot observed
4 days	PW 8	No rot observed
	NCPPB 2993	No rot observed
	C79	No rot observed
	Sterile Saline	No rot observed
7 days	PW 8	Yellowing at inoculation sites
	NCPPB 2993	Yellowing at inoculation sites
	C79	Yellowing at inoculation sites
	Sterile Saline	No rot observed
14 days	PW 8	Yellowing at inoculation sites
	NCPPB 2993	Yellowing at inoculation sites
	C79	Yellowing at inoculation sites
	Sterile Saline	No rot observed
21 days	PW 8	Yellowing at inoculation sites
	NCPPB 2993	Yellowing at inoculation sites
	C79	Yellowing at inoculation sites
	Sterile Saline	No rot observed
28 days	PW 8	Upon dissection: discoloration and rot of inner scales, strongest at inoculum sites
	NCPPB 2993	Upon dissection:strong discoloration and rot of inner scales, strongest at inoculum sites
	C79	Upon dissection: some discoloration of inner scales, stronger at inoculum sites
	Sterile Saline	No rot observed

3.3.2 Quantitative test of onion maceration

Non sterile onion

No results could be recorded due to high levels of fungal growth over all test plates.

Sterile onion

These are shown in Tables 3.4 and 3.5 shown overleaf. The growth of the test isolate on the plates in the spot test or within the wells is indicated by += growth supported , or - = growth inhibited

Table 3.4 Quantitative assay of onion maceration: well test with sterile onion

% Onion	Isolate	Clearance Zone (mm)	Growth + /-	Comments
1%	C23	0	+	Concentration of onion too low to observe clearance
	NCPPB 2993	0	+	
	PW8	0	+	
	Ps. aeruginosa PAO 2002/RP1	0	+	
	Sterile Saline	0	-	
2 %	C23	0	+	Concentration of onion too low to observe clearance
	NCPPB 2993	0	+	
	PW8	0	+	
	Ps. aeruginosa PAO 2002/RP1	0	+	
	Sterile Saline	0	-	
5%	C23	0	+	Concentration of onion too low to observe clearance
	NCPPB 2993	10	+	
	PW8	0	+	
	Ps. aeruginosa PAO 2002/RP1	0	+	
	Sterile Saline	0	-	
10%	C23	11	+	Clearance observed but with difficulty
	NCPPB 2993	16	+	
	PW8	12	+	
	Ps. aeruginosa PAO 2002/RP1	0	+	
	Sterile Saline	0	-	
25%	C23	12	+	Reasonable clearance clear, particularly NCPPB 2993
	NCPPB 2993	19	+	
	PW8	13	+	
	Ps. aeruginosa PAO 2002/RP1	0	+	
	Sterile Saline	0	-	
50%	C23	-	+	Onion too thick to observe any clearance
	NCPPB 2993	-	+	
	PW8	-	+	
	Ps. aeruginosa PAO 2002/RP1	-	+	
	Sterile Saline	-	-	

Table 3.5 Quantitative assay of onion maceration: spot test with sterile onion

% Onion	Isolate	Clearance Zone (mm)	Growth + /-	Comments
1%	C23	0	+	Concentration of onion too low to observe clearance
	NCPPB 2993	0	+	
	PW8	0	+	
	Ps. aeruginosa PAO 2002/RP1	0	+	
	Sterile Saline	0	-	
2 %	C23	0	+	Concentration of onion too low to observe clearance
	NCPPB 2993	0	+	
	PW8	0	+	
	Ps. aeruginosa PAO 2002/RP1	0	+	
	Sterile Saline	0	-	
5%	C23	0	+	Concentration of onion too low to observe clearance
	NCPPB 2993	0	+	
	PW8	0	+	
	Ps. aeruginosa PAO 2002/RP1	0	-	
	Sterile Saline	0	-	
10%	C23	10	+	Clearance observed but with difficulty
	NCPPB 2993	15	+	
	PW8	10	+	
	Ps. aeruginosa PAO 2002/RP1	0	-	
	Sterile Saline	0	-	
25%	C23	10	+	Clearance observed but with difficulty
	NCPPB 2993	16	+	
	PW8	10	+	
	Ps. aeruginosa PAO 2002/RP1	0	-	
	Sterile Saline	0	-	
50%	C23	-	+	Onion too thick to observe any clearance
	NCPPB 2993	-	+	
	PW8	-	+	
	Ps. aeruginosa PAO 2002/RP1	-	-	
	Sterile Saline	-	-	

3.4 Discussion

3. 4.1 *In vitro* onion maceration

A range of differing abilities to cause rot or maceration of onion tissue *in vitro* was found amongst the *B. cepacia* isolates tested. Some of the isolates, including those obtained from the NCPPB caused a high level of maceration accompanied by a yellow-brown discoloration (Lelliott and Stead 1987). An intense, distinctive 'sour' smell was associated with these isolates. Other strains produced a less pronounced rot through to no discernible rot. These differences are not surprising, as great variations in onion pathologenicity between *B. cepacia* strains have been described (Gonzalez and Vidaver 1979, Yohalem and Lorbeer 1994, Govan *et al* 1996).The results obtained (Table 3.1) generally, with some exeptions, confirm that clinical strains of *B .cepacia* are weak pathogens of onion (Gonzalez and Vidaver 1979, Yohalem and Lorbeer 1994). However two isolates from the Edinburgh CF centre, A548 and A562, caused high levels of onion pathology. Clinical isolates from this centre have previously been found to cause strong maceration of onion tissue (Butler et al 1995).A great deal of variation was also seen in environmental isolates. NCPPB strains caused high levels of onion maceration, as did four isolates from rivers and soils in the South Wales area: PW17, PW6, PW20 and PW11. Two other South Wales environmental isolates caused a moderate degree of rot, along with two strains from the NCIMB. All other isolates produced either low levels or no rot. It is also worth noting that the isolate PW8, obtained from rotten onion caused little rot *in vitro.*

The *Burkholderia gladioli* p.v. *allicola* strain, NCPPB 2478, also caused a high degree of onion maceration, though the nature of the rot was unlike that of *B. cepacia* as there was little discoloration or 'sour' odour. As described by Lelliott and Stead, the rot

resulted in a 'glassy' appearance. One environmental isolate, PW20, showed a similar appearance, suggesting it may be a *B. gladioli* strain rather than *B .cepacia* as typed.

The 'onion slice host test' appeared to be a more effective method than 'whole onion host tests' in terms of speed of result and the ease with which results are obtained . Using the onion slice method , results were clearly observable in three days and could be easily determined. The whole onion test results were less clear as the onion had to be cut in half to assess the amount of rot to the inner scales. An incubation period of two to three weeks was required to obtain positive results.

Onions sterilised by autoclaving in a jar showed the same properties as untreated onions in host tests. The fact that the use of autoclaved onions made little difference to host tests was a considerable advantage in the development of a quantitative test of onion maceration , as discussed below; and in selective media (Chapter 2).

3.4.2 Development of a quantitative assay of onion maceration

The use of non-sterile onions in the proposed quantitative assay was of little value as there was a large amount of fungal overgrowth in all test plates. Such growth was not found in the plates produced with autoclaved onion homogenate. In both the well and spot tests the best observable clearance of onion tissue was in plates containing 10 to 25% onion homogenate. Below this level the concentration of onion was too low to see any clearance, above this the concentration appeared too high to be cleared by the test isolates. Even at the levels of 10 to 25% it was difficult to see the clearance of onion. Staining plates with iodide, to stain any starch present in the onion, did not improve the visibility of onion clearance, the addition of a non-specific dye to the onion homogenate or agar may possibly improve the visualisation of clearance.

All *B. cepacia* strains tested cleared at least some onion homogenate in the test plates. The strongly phytopathogenic NCPPB 2993 cleared a fairly large zone of up to 19 mm around both spot and well tests. However the more weakly pathogenic isolates C23 and PW8 only cleared a small zone of around 10 to 12 mm. This is only slightly larger than the size of the well (8mm) or the size of bacterial growth in the spot test. The controls used caused no clearance of onion homogenate at any onion concentration. At the higher concentrations of onion, (10%) or more, growth of the *Ps. aeruginosa* controls were inhibited, particularly in the spot test. This showed that the test was specific for *B. cepacia* against *Ps. aeruginosa* and also demonstrated the possibility of the use of onion as a selective agent for *Burkholderia*.

The test appears to have some specificity and shows differentiation between weakly and strongly phytopathogenic *B. cepacia* strains. To quantitatively assess the potential of such an onion agar test a wider range of *B. cepacia* isolates with differing onion macerating properties would need to be tested. Nevertheless, the test shows some promise as a quantitative assay of onion maceration, but the use of a scoring system for the onion slice host test makes it a semi-quantitative assay, and may make the development of a quantitative assay unnecessary due to the ease of conducting such host tests.

Chapter 4 Antibiotic resistance and susceptibility of *Burkholderia cepacia*

4.1 Introduction

B. cepacia is resistant to many antimicrobial drugs. Determination of the antibiotic resistance profile or 'antibiogram' and the Minimum Inhibitory Concentration (MIC) of antibiotics for each isolate would be important both in the characterisation of the phenotypes of clinical and environmental strains, and for their use in further experimental work such as defining the role of antibiotic resistance plasmids. A number of methods may be used to determine antibiotic resistance profiles and MIC values (Vandepitte *et al* 1991). These include disc diffusion tests, liquid media susceptibility tests and solid media tests

4.1.1 Kirby-Bauer disc diffusion test

The principle of disc diffusion tests is relatively simple. The efficacy of an antibiotic against a bacterium is determined by placing a reservoir of an antibiotic on an agar plate evenly seeded by bacteria. The antibiotic diffuses from the reservoir becoming less concentrated the further it migrates from the source . The antibiotics' efficacy is determined by the size of the zone of bacterial growth is inhibition. Generally the more effective the antibiotic the larger the zone of inhibition (Acar and Goldstein 1985, Vandepitte *et al* 1991). In disc diffusion tests the antibiotic reservoirs are paper discs impregnated with known concentrations of antibiotics.

The Kirby-Bauer disc diffusion test was developed in the 1960s (Vandepitte *et al* 1991). It was selected as the method of choice for antibiotic sensitivity testing by the World Health Organisation (WHO) in 1977 as it is reproducible and technically simple (Vandepitte *et al* 1991). The method involves seeding of Mueller Hinton agar plates with

a bacterial suspension prepared to a standard turbidity or concentration. Antibiotic discs are then placed onto the surface of the plate with a sterile needle, forceps, or a disc-dispensing device. Plates are the incubated and zones of inhibition measured. The zones are compared to standard published values. The results are reported as resistant, susceptible or of intermediate susceptibility. In some laboratories, particularly in the UK and Europe, Diagnostic Sensitivity Test Agar (DST Agar) has been employed. This is since the emergence of BSE, as Mueller Hinton agar, which contains bovine components has generally been discontinued on safety grounds.

The amount of antibiotic in discs and interpretation of zones of inhibition is based upon an approximately linear relationship between MIC and zone size.To determine the sizes of zones obtained with resistant and susceptible bacteria, large numbers of bacterial strains have MIC determined by a broth dilution method, and l the diameters of zone of inhibition are found. The disc concentration used is based on the MIC values to range of isolates and species. These results are plotted on a regression line with MIC on the y-axis, and zone diameter on the x-axis. Susceptible (S), resistant (R) and intermediate (I) values are then determined by reading the diameters from the graph equivalent to the upper and lower critical breakpoint serum concentrations of the drug in clinical use. Bacteria are reported as susceptible to an antibiotic when they are likely to respond to treatment with thc drug. Intermediate susceptibility applies to bacteria that are 'moderately' susceptible to an antibiotic at the concentration tested. If the antibiotic is of low toxicity it may be effective at a higher concentration. The results of these tests may be used by clinicians to decide on the appropriate antimicrobial therapy, or used in the epidemiological surveillance of antibiotic resistance.

4.1.2 Determination of minimum inhibitory concentration (MIC) by agar dilution

Agar media are commonly used to determine the MIC of antibiotics by use of a series of plates containing varying concentrations of antibiotics. Such procedures are known as Agar Dilution Tests. (Barry 1985). Many methods may be employed to determine MIC including broth dilution, agar diffusion tests and gradient plates. Agar dilution techniques have a number of advantages, particularly over broth methods. The use of replicating inoculating devices such as Multipoint Inoculators allow large numbers of samples to be tested together quickly and accurately with consistency of inoculum size. Supplements such as blood required for fastidious organisms can easily be added.. This may not be the case using broths where supplements may cause the media to become cloudy, making interpretation of end points difficult. Any contamination of the test isolate may be more easily detected on agar plates as differing colony morphologies may result. In addition the majority of further experiments to be conducted involving antibiotics such as mating experiments will be primarily conducted on agar, so there would be greater consistency if MIC values were determined on agar media.

The basis of Agar Dilution techniques is production of a series of Mueller Hinton or DST agar plates containing a range of concentrations of the antibiotic to be tested. Overnight cultures of the test isolates are inoculated onto the surface of the agar plates , which are then incubated. The MIC is recorded as the lowest concentration of antibiotic that inhibits the growth of the isolate not including the growth of a single colony or hazy growth at the inoculum site .

In both the determination of MIC or Disc Diffusion tests it is necessary to include suitable reference strains as quality controls. Organisations such as the WHO or the National Committee for Clinical Laboratory Standards publish lists of suitable control isolates.

4.2 Materials and methods

Materials

i. Chemicals

Antibiotic discs (Oxoid, Unipath, Basingstoke, Hants)

Ceftazidime, gentamicin sulphate, cefuroxime sodium, tobramycin, amikacin and sulphamethoxazole (Sigma Chemical Co., Poole, Dorset) made up to appropriate stock solutions in double distilled water (or methanol if not water soluble) and filter sterilised.

ii.Strains

B. cepacia strains (as described in Chapter2)

E. coli NCTC 10418 control culture

iii. Media

DST agar plates (Oxoid CM26, Unipath Ltd, Basingstoke, Hants.)

20 ml aliquots molten DST agar

iv. Solutions

MacFarland 0.5 turbidity standard (BioMerieux, Marcy l'Etoile, France)

10 ml aliquots of sterile 0.85% w/v saline

v. Equipment

Oxoid antibiotic disc dispenser (Unipath, Basingstoke, Hants)

Multi-point inoculator (Mast Diagnostics, Bootle, Merseyside)

Rule or measuring calipers (mm scale)

vi. Disposable consumables

Sterile cotton wool swabs

Sterile plastic 90 mm Petri dishes

Methods

i. Kirby-Bauer disc diffusion test

The antibiotic discs used were based on those recommended by the WHO and by Acar and Goldstein (1985) for *Pseudomonas* species, and on their availability in the laboratory. The WHO recommends piperacillin, tobramycin and gentamicin as first choice. Other antimicrobials tested include ceftazidime, tetracycline and azlocillin as recommended by Acar and Goldstein (1985). The full list of antibiotics used along with their interpretative zone diameters are shown in Table 4.1.

An inoculum of each isolate to be tested was prepared by picking between 3 to 6 colonies from an overnight culture on nutrient agar at 35 °Cand suspending the colonies in 10ml of sterile saline to a MacFarland Turbidity Standard of 0.5. The DST plates were then inoculated to give a lawn of growth. A sterile cotton swab was dipped into the prepared inoculum, the excess was removed by pressing the swab against the side of the container; and then the swab was then streaked over the surface of the plate three times, rotating 60° between each application. The inoculated plates were allowed to dry for 10 to 20 mins before antibiotic discs were applied to the surface of the plates using an Oxoid Disc Dispenser. Plates were then incubated at 35°C for 24 to 48 h. Zones of inhibition were recorded, the diameters measured either with a rule on the underside of the plate or by using a pair of callipers. The diameters recorded were compared with published interpretative tables to determine whether the isolate is resistant or susceptible to the antibiotic (Acar and Goldstein 1985, Vandepitte *et al* 1991).

ii. MIC determination by agar dilution method

Molten 20 ml aliquots of DST agar were maintained at 50°C in a waterbath. Antibiotic stock solutions were added to the molten agar to give final concentrations of 0, 1, 2, 5,

10, 20, 25, 50, 100, 250 and 500 μg ml^{-1}. The agar containing antibiotics was then poured into appropriately labelled petri dishes. The plates were then allowed to set and dry for 4 h, or overnight, prior to use. 1 ml of overnight broth cultures of isolates to be tested were pipetted into the sterile PTFE dish of the Multipoint Inoculator. This was placed onto the stage of the inoculator and the sterile inoculating pins placed into their holder. The first plate to be inoculated, with lid removed, was placed onto the stage of the inoculator that was then operated by means of a foot switch. This was repeated for each of the test plates. After inoculation the plates were incubated for 24 to 48 h at 35°C. The plates were then read, the MIC being recorded as the plate with the lowest concentration of antibiotic that inhibits growth of the isolate not including hazy growth or a single colony at the inoculum site.

4.3 Results

4.3.1 Kirby-Bauer disc diffusion test for antibiotic resistance/susceptibility of *B. cepacia* strains

The antimicrobial content and zone interpretation values are shown over in Table 4.1. The results obtained by this method are shown in Tables 4.2, 4.3 and 4.4 . Tables 4.5, 4.6 and 4.7 show the percentages of isolates tested found resistant (R) ,susceptible (S) or of intermediate (I) susceptibility to each antibiotic. Where susceptibility of an isolate to an antibiotic was not tested, or no readable value was obtained are shown by a dash (-) .

Table 4.1 **Antimicrobial agents used in modified Kirby -Bauer testing of *B. cepacia* isolates: Disc antimicrobial content and zone interpretative standards (WHO)**

Antimicrobial Agent	Disc Content	Code	Zone Diameter (mm)		
			Resistant (R)	Intermediate (I)	Susceptible (S)
amoxocillin	25 µg	AML	<13	14-20	≤ 21
azlocillin	75 µg	AZL	< 9	10-18	≤ 19
ceftazidime	30 µg	CAZ	< 14	15-20	≤ 21
cefuroxime	30 µg	CXM	< 13	14-21	≤ 22
erythromycin	15 µg	E	< 13	14-17	≤18
gentamicin	10 µg	CN	< 12	13-14	≤ 15
novobiocin	5 µg	NV	< 15	16-20	≤ 21
penicillin G	10 units	P	< 11	12-21	≤22
piperacillin	75 µg	PRL	< 14	15-17	≤ 18
sulphamethox-azole	25 µg	RL	< 13	14-16	≤ 18
tetracycline	30 µg	TET	< 14	15-19	≤ 20
tobramycin	10 µg	TOB	< 12	13-14	≤ 15

Acar and Goldstein (1985), and Vandepitte *et al* (1991)

Table 4.2 Antibiotic susceptibility of clinical *B. cepacia* isolates by modified Kirby-Bauer disc diffusion method

Isolate	Antibiotic											
	TE	CN	AZL	TOB	P	CAZ	AML	NV	CXM	PRL	E	RL
C5	I	R	R	R	R	R	R	R	S	R	R	S
C11	R	S	S	S	R	R	I	R	I	S	R	S
C23	R	S	S	R	R	R	R	R	S	S	R	S
C96	R	R	R	R	R	R	R	R	S	R	R	S
C59	R	R	R	R	R	R	R	R	R	R	R	R
C1	R	R	R	R	R	I	R	R	R	R	R	S
C49	R	R	R	R	R	R	R	R	S	-	R	S
C51	R	R	R	R	R	I	I	R	S	R	R	S
C79	R	S	S	S	R	S	R	R	R	S	R	I
C81	R	R	R	R	R	I	R	R	R	R	I	S
C93	R	R	R	R	R	S	R	R	S	R	R	S
C95	R	R	R	R	R	S	R	R	S	R	R	S
C116	I	R	R	R	R	I	R	R	R	R	R	R
C187	R	R	R	R	R	R	R	R	S	R	R	S
C190	R	R	R	R	R	I	R	R	S	R	R	S
C205	R	R	R	R	R	R	R	R	S	R	R	S
A562	R	R	I	R	R	I	R	R	R	I	R	R
A548	R	R	S	R	R	S	R	R	R	S	R	-
C1860	R	R	S	R	R	S	R	R	R	S	R	R
C1858	R	R	S	R	R	S	R	R	R	S	R	R
J543	R	R	I	R	R	S	R	R	R	I	R	R
J478	R	R	S	R	R	S	R	R	R	S	R	R
D2	I	R	I	R	R	R	R	R	I	I	R	-
D3	R	R	S	R	R	R	R	R	R	S	R	-
D4	R	R	S	R	R	R	R	R	R	-	R	-
D5	R	R	S	R	R	S	R	R	R	S	R	-
D6	R	R	S	S	R	S	R	R	R	S	R	-

Table 4.3 **Antibiotic susceptibility of environmental *B. cepacia* isolates by modified Kirby-Bauer disc diffusion method**

Isolate	Antibiotic											
	TE	CN	AZL	TOB	P	CAZ	AML	NV	CXM	PRL	E	RL
J2552	R	S	S	S	R	S	R	R	S	S	R	R
NCIMB 9085	R	R	S	R	R	S	R	R	S	S	R	-
NCIMB 9088	R	R	S	I	R	S	R	R	I	S	R	-
NCIMB 9092	R	R	S	R	R	S	R	R	R	S	R	-
NCPPB 2993	R	R	S	I	R	S	R	R	R	S	R	-
NCPPB 3480	R	R	S	R	R	S	R	R	R	S	R	-
PW1	R	R	S	R	R	S	R	R	R	S	R	-
PW2	R	S	I	S	R	I	R	R	R	S	R	-
PW3	R	S	I	S	R	S	R	R	R	S	R	-
PW4	R	R	I	R	R	R	R	R	R	S	R	-
PW5	R	S	S	S	R	S	R	R	R	S	R	-
PW6	R	S	S	S	R	I	R	R	R	S	R	-
PW7	R	S	S	S	R	S	R	R	R	S	R	-
PW8	R	S	S	S	R	R	R	R	R	S	R	-
PW9	S	S	S	R	R	S	R	R	R	S	R	-
PW10	I	S	S	S	R	S	R	R	R	S	R	-
PW11	R	S	I	S	R	R	R	R	R	S	R	-
PW12	I	S	S	S	R	S	R	R	R	S	R	-
PW13	I	S	S	S	R	S	R	R	R	S	R	-
PW14	R	S	S	S	R	-	R	R	R	S	R	-
PW15	R	R	S	R	R	S	R	R	I	S	R	-
PW16	R	R	S	R	R	I	R	R	R	S	R	-
PW17	S	S	S	S	R	I	R	R	R	S	R	-
PW18	I	S	S	S	R	S	R	R	R	S	R	-
PW19	I	S	S	S	R	S	R	R	R	S	R	-
PW20	R	R	S	R	R	S	I	R	S	S	R	-
PW21	R	R	S	S	R	-	R	R	S	S	R	-

Table 4.4 Antibiotic susceptibility of control and *B. gladioli* isolates by modified Kirby-Bauer disc diffusion method

Isolate	Antibiotic											
	TE	CN	AZL	TOB	P	CAZ	AML	NV	CXM	PRL	E	RL
B. gladioli NCPPB 2478	R	S	S	S	R	S	R	R	R	S	R	-
E .coli NCTC 10148	R	S	S	S	R	S	I	R	S	S	R	-

Table 4.5 % clinical *B. cepacia* isolates resistant/susceptible to a range of antibiotics by modified Kirby-Bauer disc diffusion test

	Antibiotic											
	TE	CN	AZL	TOB	P	CAZ	AML	NV	CXM	PRL	E	RL
% of susceptible isolates	0	11	41	11	0	37	0	0	37	40	0	62
% of resistant isolates	89	89	48	89	100	41	96	100	56	48	96	35
% of isolates of intermediate susceptibility	11	0	11	0	0	22	4	0	7	12	4	5

Table 4.6 **% environmental *B. cepacia* isolates resistant/susceptible to a range of antibiotics by modified Kirby-Bauer disc diffusion test**

	Antibiotic											
	TE	CN	AZL	TOB	P	CAZ	AML	NV	CXM	PRL	E	RL
% of susceptible isolates	7	59	85	59	0	72	0	0	11	100	0	-
% of resistant isolates	74	41	0	33	100	12	100	100	78	0	100	-
% of isolates of intermediate susceptibility	19	0	15	8	0	16	0	0	11	0	0	-

Table 4.7 % Total *B. cepacia* isolates resistant/susceptible to a range of antibiotics by modified Kirby-Bauer disc diffusion test

	Antibiotic											
	TE	CN	AZL	TOB	P	CAZ	AML	NV	CXM	PRL	E	RL
% of susceptible isolates	4	35	63	19	0	54	0	0	34	71	0	63
% of resistant isolates	81	65	34	61	100	27	98	100	67	23	98	36
% of isolates of intermediate susceptibility	15	0	13	4	0	19	2	0	9	6	2	3

4.3.2 MIC determination by Agar Dilution

MIC values, expressed as μg ml^{-1}, obtained by Agar Dilution in DST Agar are shown on Table 4.8, Table 4.9 and Table 4.10 . Where the antibiotic was not tested against the isolate a dash (-) is shown.

Table 4.8 MIC (μg ml^{-1}) of antibiotics to *B. cepacia* clinical isolates by the Agar Dilution Multi Point Inoculator method

Isolate	ceftazidime	gentamicin	piperacillin	tobramycin	amikacin	sulphameth-oxazole	cefuroxime sodium
C5	10	50	100	25	25	10	10
C11	10	50	25	25	25	10	5
C23	10	50	25	25	25	5	5
C96	10	50	25	25	25	5	5
C59	10	50	25	25	25	25	5
C1	10	50	100	25	25	5	5
C49	10	50	250	25	25	25	5
C51	10	50	25	25	25	5	25
C79	10	25	25	25	25	25	5
C81	10	25	25	25	25	25	5
C93	10	250	25	50	100	500	5
C95	10	50	25	25	25	5	5
C116	10	50	25	25	25	25	5
C187	5	100	25	50	50	5	25
C190	10	50	1	25	25	5	25
C205	10	50	25	25	25	25	5
A562	5	100	10	100	250	250	10
A548	1	>500	5	500	500	250	10
C1860	5	100	10	100	250	500	-
C1858	5	250	10	250	500	250	-
J543	5	100	5	100	100	50	10
J478	5	50	10	25	100	500	25
D2	10	50	100	-	-	-	-
D3	5	500	100	-	-	-	-
D4	5	500	5	-	-	-	-
D5	1	250	100	-	-	-	-
D6	5	10	5	-	-	-	-

Table 4.9 MIC (μg ml^{-1}) of antibiotics to *B. cepacia* environmental isolates by the Agar Dilution Multi Point Inoculator method

Isolate	ceftazidime	gentamicin	piperacillin	tobramycin	amikacin	sulphameth-oxazole	cefuroxime sodium
J2552	5	25	5	100	50	250	5
NCIMB 9085	5	250	5	100	10	250	25
NCIMB9088	1	100	5	100	50	50	5
NCIMB 9092	5	25	1	10	25	500	5
NCPPB 2993	5	100	10	100	100	500	25
NCPPB 3480	5	250	10	100	100	250	10
PW1	1	250	10	-	-	-	-
PW2	1	1	10	-	-	-	-
PW3	1	1	10	-	-	-	-
PW4	25	100	10	-	-	-	-
PW5	5	1	1	-	-	-	-
PW6	5	1	1	-	-	-	-
PW7	1	5	10	-	-	-	-
PW8	-	-	-	-	-	-	-
PW9	1	10	>250	-	-	5	-
PW10	-	-	-	-	-	-	-
PW11	5	1	10	-	-	250	-
PW12	1	1	25	-	-	250	-
PW13	1	1	10	-	-	250	-
PW14	1	1	10	-	-	250	-
PW15	-	-	-	-	-	-	-
PW16	-	-	-	-	-	-	-
PW17	5	1	1	-	-	5	-
PW18	1	5	25	-	-	250	-
PW19	1	1	1	-	-	250	-
PW20	1	1	10	-	-	250	-

Table 4.10 MIC (μg ml^{-1}) of antibiotics to *B .gladioli* and *Ps. aeruginosa* isolates by the Agar Dilution Multi Point Inoculator method

Isolate	ceftazidime	gentamicin	piperacillin	tobramycin	amikacin	sulphameth-oxazole	cefuroxime Sodium
B. gladioli NCPPB 2478	5	1	5	1	1	>500	1
Ps. aeruginosa Pao1 R300B	1	1	5	1	5	>500	>100
Ps. aeruginosa Pao1 pQM1 R300B	1	1	5	1	5	>500	>100
Ps. aeruginosa Pao2002 RP1	1	1	>250	5	5	100	>100

4.4 Discussion

4.4.1 Antibiotic resistance/susceptibility

The Kirby-Bauer method proved to be a reliable method of determining antibiotic resistance or susceptibility of *B. cepacia* isolates. Isolates run as repeats, NCPPB 2993 and C23, had diameter variations of only 1mm between each set of plates tested. Results obtained were broadly as expected. Virtually all isolates were resistant to novobiocin, amoxycillin, penicillin G and erythromycin (Table 4.7). Many isolates, were resistant to tetracycline (89% of clinical isolates, 74% of environmental isolates) and the aminoglycosides tobramycin and gentamicin (89% of clinical isolates), (Table 4.6). The number of isolates resistant to the antipseudomonal ureidopenicillins piperacillin and azlocillin, ceftazidime and sulphamethoxazole was found to be more variable. Environmental strains were found to be particularly susceptible to azlocillin (85% susceptible), piperacillin (100% susceptible) and ceftazidime (72% susceptible), (Table 4.5).

These results obtained compare broadly to those of Santos-Ferreira *et al* (1985) for nosocomially acquired strains of *B. cepacia* from non-CF patients, and Bhakta *et al* (1992) for *B. cepacia* isolates from CF patients.(Table 4.11)

Table 4.11 % of *B. cepacia* isolates resistant/susceptible to a range of antibiotics in this and previous studies

	Santos-Ferreira *et al* (1985)		Bhakta *et al*(1992)		Environmental isolates this study		Clinical isolates this study		All isolates this study	
Antibiotic	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**
cefuroxime	100	0	-	-	78	11	56	37	67	33
gentamicin	93	3	-	-	11	49	89	11	65	35
tobramycin	90	7	93	5	33	59	89	11	61	19
tetracycline	100	0	-	-	74	7	89	0	81	4
sulphonamides*	4	96	-	-	-	-	35*	62*	36*	63*
novobiocin	0	100	-	-	100	0	100	0	100	0
piperacillin	<1	85	11	60	0	100	48	40	23	71
azlocillin	0	67	-	-	0	85	48	41	34	63
ceftazidime	0	67	9	81	12	72	41	37	27	54

* Results for sulphamethoxazole

The numbers of isolates resistant to tobramycin reported by Bhakta *et al* (1992) compares closely with those obtained in this study , (93% and 89% resistant respectively). However levels of resistance found for piperacillin (48%) and ceftazidime (41%) in clinical isolates are considerably higher than the levels recorded by Bhakta *et al.* (1992) . This is possibly due to increased use of these antibiotics in the treatment of infections in CF, particularly in the UK.

The numbers of isolates resistant to aminoglycosides and tetracycline reported by Santos-Ferreira *et al* (1985) were similar to those found amongst our isolates. The main differences found were in the resistance levels to novobiocin and cefuroxime. All isolates tested were found to be resistant to novobiocin, whereas Santos-Ferreira *et* al found all isolates to be susceptible to this drug. Conversely all isolates were found to be resistant to cefuroxime by Santos-Ferreira *et al,* (1985) compared with susceptibility levels of 34% in our isolates. The pattern of resistance to azlocillin, piperacillin and ceftazidime was similar to that found in the environmental isolates, with little resistance to these drugs overall.

Distinct patterns of resistance were seen amongst the clinical isolates. Generally isolates from Cardiff CF patients were resistant to azlocillin, piperacillin and ceftazidime but susceptible to sulphamethoxazole. Isolates from Edinburgh and Strasbourg CF centres were resistant to sulphamethoxazole but more susceptible to azlocillin, piperacillin and ceftazidime. Such patterns may be due to the development of acquired resistance to such drugs, possibly due to therapy with ureidopenicillins and

ceftazidime being predominantly used in Cardiff, and sulphamethoxazole as a component of co-trimoxazole with trimethoprim more commonly used at other centres.

4.4.2 MIC determination

Although the Agar Dilution method was reasonably quick and easy to perform, a number of problems were found. Determination of the end point was somewhat difficult . Judging whether there is growth , hazy growth or a single colony is rather subjective unlike the determination of a zone size or an absorbance value. Variability was also a problem. as repeated isolates could vary considerably in MIC value. For example repeats of C23 varied in MIC values between 25 to 100μg ml^{-1} for piperacillin.

Overall the results obtained were again comparable with published values. Bhakta *et al* (1992) described a range of MIC's in *B. cepacia* CF isolates of 4 to 32 μg ml^{-1} for ceftazidime as compared with 1 to 10 μg ml^{-1} for CF isolates tested, 16 to 128 μg ml^{-1} for piperacillin compared with 5 to 250 μg ml^{-1}, 1 to >64 μg ml^{-1} for amikacin as compared with 25 to 500 μg ml^{-1} and 0.25 to >16 μg ml^{-1} for tobramycin compared with 25 to 500 μg ml^{-1}. It is notable that very high MIC levels were obtained for aminoglycoside drugs with some isolates. Bhakta *et al* (1992) also found that the majority of isolates, some 90 %, had MIC levels for piperacillin of 64 μg ml^{-1} or below compared with a level of 25 μg ml^{-1} for most of the CF isolates tested in this study. An earlier study of clinical and environmental isolates found MIC levels of less than 5 μg ml^{-1} to 640 μg ml^{-1} for gentamicin (Gonzalez and Vidaver 1979). This compares well to the MIC levels of 1 μg ml^{-1} to over 500 μg ml^{-1} found in the isolates in this study.

The MIC's described by Santos-Ferreira *et al* (1985) for nosocomial *B. cepacia* isolates also compare well to the results obtained. A range of 0.125 to 16 μg ml^{-1} was found for ceftazidime, with most isolates (around 80 %), having a MIC of 1 or less. The results obtained were in the range of 1 to 25μg ml^{-1}, with most CF isolates in the range of 5 to 10 μg $ml^{-1}$and most environmental isolates were in the range of 1 to 5 μg ml^{-1}. Santos-Ferreira *et al* (1985) found a MIC range of 0.125 μg $ml^{-1}$to 32 μg $ml^{-1}$for piperacillin, with the majority, over 90%, having a value of 8 μg ml^{-1}or less. This closer to the MIC range for environmental isolates, 1 to 10 μg ml^{-1} than that of CF isolates, a range of 1 to 250μg ml^{-1}, with the majority having an MIC of 25 μg ml^{-1}. This level being higher than that of the nosocomial isolates.

A notable point is that using British Society for Antimicrobial Chemotherapy Working Party breakpoints few of our isolates would be regarded as resistant to piperacillin at the upper breakpoint of 64 mg l^{-1}(or 64μg ml^{-1})(British Society for Antimicrobial Chemotherapy 1991). Only 6 of 27 CF isolates (23 %) and 1 of 23 environmental isolates (4 %) would be resistant at the 64 μg ml^{-1} breakpoint, compared with 20 of 42 (42 %) of CF isolates and 1 of 25 environmental isolates (4 %) found resistant by Butler *et al* (1995).

As with the Kirby-Bauer test results patterns of MIC levels could be seen amongst the CF isolates. Generally, the main exception being C93, Cardiff isolates have MIC levels to ceftazidime of around 10 μg ml^{-1} piperacillin 25 μg ml^{-1}, gentamicin 50μg ml^{-1}, tobramycin 25 μg ml^{-1}, amikacin 25 μg $ml^{-1}$and sulphamethoxazole 5 to 25 μg ml^{-1}.

Isolates from Edinburgh and Strasbourg CF centres have lower MIC levels for ceftazidime 1to 5 μg ml^{-1}, and piperacillin, 5 to 10 μg ml^{-1}, but much higher MIC levels for tobramycin, gentamicin, amikacin and sulphamethoxazole. In some cases these values exceed 500 μg ml^{-1}

4.4.3 Resistance in environmental and clinical strains

Generally environmental isolates appear to be less resistant to antimicrobials than clinical isolates from CF patients. This seems particularly true of piperacillin, azlocillin and ceftazidime with most environmental isolates susceptible to these drugs. A range of resistance levels to aminoglycosides was found amongst environmental isolates. Although some isolates had high MIC levels of 250 μg ml^{-1} for gentamicin, many had low levels of 1to 5 μg ml^{-1} with 89 % being susceptible compared with only 11 % of CF isolates being susceptible. Butler *et al* (1995) found environmental isolates were more susceptible to antibiotics than CF isolates. Determination of MIC by Agar Dilution showed the vast majority of environmental isolates having MIC levels below breakpoint levels for piperacillin, meropenem and ceftazidime whereas much higher numbers of clinical isolates were above these levels.

Levels of resistance amongst non-CF *B. cepacia* clinical isolates are considerably lower and more comparable to environmental isolates. Gonzalez and Vidaver (1979) found little difference in MIC levels between environmental and non-CF clinical isolates, and the results of Santos-Ferreira *et al* (1985) more closely resemble the values obtained for the environmental isolates than those of the CF isolates. Although it should be remembered that *B. cepacia* is innately resistant to many antibiotics, the aggressive

antimicrobial chemotherapy employed in CF respiratory infections would obviously place selective pressure on *B. cepacia* infecting CF patients. This could have two effects; firstly selecting for the more resistant strains of *B. cepacia* and eliminating any with low resistance to antimicrobials and secondly, it might lead to acquired high level resistance to the antimicrobials used. Alternatively such differences may be the result of the strains infecting CF patients having fundamental differences to those in the environment or causing nosocomial infections.

4.4.4 Antimicrobial resistance/susceptibility in *B. cepacia*: General considerations

The results obtained were broadly those expected, with most isolates resistant to many antibiotics including tetracycline, erythromycin, novobiocin, penicillins and aminoglycosides with varying resistance levels to ureidopenicillins, ceftazidime and sulphamethoxazole. *B. cepacia* would also be expected to be resistant to other antibiotics not tested including polymixins such as colistin, polymixin B and ticaricillin. These drugs are employed in the *B. cepacia* selective media described by Gilligan *et al* (1985) and produced commercially by Mast Diagnostics. In the clinical testing of antibiotics it would also be important to include trimethoprim and trimethoprim-sulphamethoxazole (co-trimoxazole), often used in treatment of *B. cepacia* infections. It would also be more suitable to test a broader range of antibiotics for MIC determination, using doubling dilutions from 1056 $\mu g\ ml^{-1}$ to 1 $\mu g\ ml^{-1}$

The use of the Kirby-Bauer disc diffusion test for determining resistance or susceptibility to antibiotics proved to be convenient and reliable and although there were some problems with MIC determination by agar dilution, these tests were of

considerable value in determining the antibiotic resistance phenotypes for a range of environmental and clinical *B. cepacia* isolates. The tests defined levels of antibiotic resistance for further experiments and showed the intrinsic multiresistance to antibiotics displayed by *B. cepacia* of both environmental and clinical origin and hence the problems faced by clinicians in treating *B. cepacia* infections.

Chapter 5 Isolation and purification of plasmid DNA from *B. cepacia*

5.1 Introduction

5.1.1 Principles of plasmid DNA isolation and purification

Isolation of plasmid DNA relies upon the small size and the covalently closed circular (ccc) nature of plasmid DNA compared with the larger, less supercoiled chromosomal DNA (Sambrook *et al* 1989, Rohde 1995). All methods require the lysis of cells prior to the purification of plasmid DNA. Lysis techniques include the use of lysozyme, EDTA, detergent, boiling or treatment with alkali (Sambrook *et al* 1989).

One of the most established methods for the separation of plasmid from chromosomal DNA is equilibrium centrifugation in caesium chloride (CsCl) - ethidium bromide gradients (Sambrook *et al* 1989). CsCl gradients are produced by high-speed centrifugation of saturated solutions of CsCl, giving gradients that are dense at the bottom and less so at the top. Molecules float in the gradient according to their buoyant densities; DNA floats in the middle of a gradient, protein floats at the top and RNA pellets at the bottom. The addition of ethidium bromide allows visualisation of DNA. Ethidium bromide is a planar molecule that intercalates between bases in DNA molecules. Chromosomal DNA, nicked or open circular plasmid DNA allows the intercalation of large amounts of ethidium bromide. This decreases the buoyant density of the molecule. In contrast cccDNA in the supercoiled plasmids allows the intercalation of only small amounts of ethidium bromide. Consequently chromosomal DNA is more buoyant and floats above the plasmid DNA. Illumination with ultraviolet

(UV) light visualises DNA intercalated or stained with ethidium bromide. This allows the lower band of ccc plasmid DNA to be withdrawn from the tube with a syringe. However this method is expensive, requiring an ultracentrifuge, and time consuming.

Numerous other methods have been developed to isolate plasmid DNA. Many rely on lysis treatments that also disrupt base pairing in DNA molecules resulting in the denaturation of chromosomal DNA. ccc plasmid DNA strands are not separated as they are intertwined by high levels of supercoiling. A return to neutral, non-lytic conditions allows the plasmid DNA to return to its correct pairing accurately and rapidly (Sambrook *et al* 1989).

'Cleared lysate' methods such as those of Rodriguez and Tait (1983) and Birnboim (1983) use lysis by lysozyme, detergents or alkali to break up bacterial cells and fragment chromosomal DNA. The samples are then centrifuged to remove cell debris and the majority of chromosomal DNA. This results in a 'cleared lysate' containing plasmid DNA and any remaining chromosomal DNA. The DNA may then be precipitated by cold alcohol. Rapid boiling methods for small plasmids employ similar methods (Sambrook *et al* 1989).

The isolation method of Kado and Liu (1981) uses a high alkaline pH at a raised temperature to denature proteins and chromosomal DNA. The resulting lysate is then mixed and emulsified with a phenol/chloroform mixture. The emulsion is broken by centrifugation . The phenol/chloroform extraction results in the ccc plasmid DNA being left in the upper aqueous phase. Proteins, cell wall debris and the majority of

chromosomal DNA precipitates at the aqueous/solvent interface. The upper layer can be removed and the DNA precipitated if required.

A number of other methods including ion exchange, gel filtration and differential centrifugation with polyethylene glycol or sucrose gradients have also been utilised for plasmid isolation(Sambrook *et al* 1989). Commercial kits for plasmid purification often use a combination of production of a cleared lysate before separation of the DNA by ion exchange. Qiagen and Qiafilter systems (Qiagen 1996) use a diethylaminoethanol (DEAE) anion exchange resin. Anion exchange works by binding the negatively charged DNA to the resin. The DNA can then be eluted by use of buffer at lower pH or altered ionic strength. The elution with Qiagen kits is with a high salt buffer. Promega's 'Wizard' system employs resin slurry and a mini spin column in a similar method (Promega 1996).

Large plasmids are usually of lower copy number than small plasmids. (Sambrook *et al* 1989). A number of techniques have been used in *E. coli* to increase plasmid yield, notably growth in Terrific broth or using chloramphenicol amplification (Sambrook *et al*1989). The basis of chloramphenicol amplification is that chloramphenicol will inhibit protein synthesis and hence cell replication without inhibiting DNA synthesis. This would allow plasmid replication to continue, thereby increasing copy number (Sambrook *et al* 1989).

Agarose gel electrophoresis is the most commonly employed method for plasmid identification and characterisation . Gels are usually produced and electrophoresed

either in tris-borate (TBE) buffer or inh tris-acetate (TAE) buffer. Negatively charged DNA migrates towards the anode in an electric field. Rate of movement is based upon size (small molecules move through the gel more rapidly), and conformation (any nicked or open circular DNA would move more slowly than cccDNA) (Gartland and Gartland 1993). DNA can be visualised by staining with ethidium bromide and illuminating with UV light and photographs can be taken to give a permanent record.

Plasmid size may be estimated by running and plotting $\log_{10}$ of DNA size (in kb or MDa) of plasmids of known sizes such as those of *E. coli* V517 (Macrina *et al* 1978). against distance travelled to give a standard curve from which size can be calculated. This method has been applied successfully with several species, notably *Salmonella* where it has formed the basis of typing by plasmid profiling (Threlfall and Frost 1990). A major problem with this method is the curved line obtained resulting in inaccurate results. Greater accuracy can be obtained by plotting $\log_{10}$ molecular size against $\log_{10}$ relative mobility (Meyers *et al* 1976). This may be further improved by multiple regression analysis of the $\log_{10}$ molecular size against $\log_{10}$ relative mobility and the reciprocal square root of the relative mobility (Rochelle *et al* 1985). This was found to give accurate estimations of plasmid size over a large range and with a variety of agarose strengths. Alternatively plasmid DNA may be digested into linear fragments with restrcition endonucleases and size compared with molecular markers (Gartland and Gartland 1993). Summation of the sizes of the fragments allows the size of the plasmid molecule to be calculated. Many workers regard this method as more accurate, particularly with large plasmids.

5.1.2 Plasmid isolation and detection in *B. cepacia*

Plasmids in *B. cepacia* have been reported as being large in size, often in excess of 200 kb (Lennon and DeCicco 1991, Bhat *et al* 1994) . A number of methods of plasmid isolation have been employed. Equilibrium centrifugation with CsCl and ethidium bromide was used to isolate antibiotic resistance plasmids in *B. cepacia* (Williams *et al* 1979, Hirai *et al* 1982), and more recently degradative plasmids (Haak *et al* 1995). Lennon and DeCicco (1991) used a modified method based on that of Birnboim (1983), including an additional phenol/chloroform extraction. Methods based on those of Kado and Liu (1981) have been used to isolate mercury resistance plasmids (Rochelle *et al* 1988) and degradative plasmids. Methods based on those of Hansen and Olsen (1978) have been also used to isolate large plasmids from *B. cepacia* (Xia *et al* 1996).

5.1.3 Methods for detection and purification of plasmid DNA used in this study

The methods employed in this study were chosen on the basis of those previously used to isolate plasmids in *B. cepacia* and those previously used in this laboratory (Duggan 1993). Methods based on those of Kado and Liu (1981), Birnboim (1983) and the modified method used by Lennon and DeCicco(1991), the cleared lysate method of Rodriguez and Tait (1983) .A number of commercially available kits were chosen for use to assess their usefulness in quickly obtaining large quantities of plasmid DNA in *B. cepacia.* Size of plasmids was estimated by comparison with the known plasmids in *E. coli* V517 (Macrina *et al* 1978) and *E. coli* 39R (Threlfall *et al* 1986)

5.2 Materials and methods

Materials

i.Chemicals

Chloroform, Molecular Biology Grade (Sigma Chemical Co., Poole, Dorset)

Equilibrated phenol, Molecular Biology Grade (Sigma Chemical Co.) and supplied isoamyl alcohol

Agarose, Molecular Biology Grade (Sigma Chemical Co.)

ii.Strains

Size reference markers:

Escherichia coli V517 containing eight plasmids of known size (Macrina *et al* 1978).

E. coli 39R861 containing four plasmids of known size (Threlfall *et al* 1986).

Pseudomonas aeruginosa PAO 2002/pQM1 containing a single large plasmid were.

All strains were maintained as frozen stocks at -70^0 C with 20% v/v glycerol. The sizes of the plasmid standards are shown in Table 5.1.

Table 5.1 **Size of plasmids in reference strains**

Strain	Plasmid	Size (kb)
E. coli V517	pVA517A	55.1
	pVA517B	7.4
	pVA517C	5.6
	pVA517D	5.2
	pVA517E	4.1
	pVA517F	3.1
	pVA517G	2.7
	pVA517H	2.1
E. coli 39R861	A	151.0
	B	65.0
	C	36.8
	D	7.1
Ps. aeruginosa PAO2002	pQM1	254.0

Other strains:

Overnight 10 ml cultures of *B. cepacia* and other species were grown in NB as previously described (Chapter 2 p.97),unless otherwise stated.

25 to 100 ml overnight cultures were grown at 37°C in an orbital incubator at 200 r.p.m for 16 h.

iii. Media

Luria Bertani (LB) broth (Appendix 1), pre-warmed to 37°C where indicated.

iv. Solutions

Lysing solution (Appendix 1)

1 mg ml^{-1} lysozyme prepared in filter sterilised distilled water (Sigma Chemical Co., Poole, Dorset)

Alkaline sodium dodecyl sulphate solution (Appendix 1)

High salt solution (Appendix 1)

Acetate MOPS buffer (Appendix 1)

Gel loading buffer (Appendix 1)

TBE buffer (Appendix 1)

TE buffer (Appendix 1)

Chloroamphenicol solution, 34 mg ml^{-1}, dissolved in ethanol (Sigma Chemical Co., Poole, Dorset)

Ethidium bromide solution, 1mg ml^{-1}, prepared in double distilled water (Sigma Chemical Co., Poole, Dorset)

Saturated sodium chloride solution: Analar grade NaCl (BDH, Poole, Dorset). dissolved in double distilled water under saturated, then sterilised by autoclaving at 121°C, 15 p.si. for 15 mins.

v. Equipment

Horizon 58 mini gel electrophoresis tank(Gibco BRL, Paisley Scotland)

Bio Rad sub-cell electrophoresis tank (Bio Rad,Hemel Hempstead, Herts.)

250–EX electrophoresis power supply (Gibco BRL, Paisley, Scotland)

Sorvall RC-5B, high speed centrifuge and centrifuge tubes

MSE Microcentaur microcentrifuge

Vi. Disposable consumables

1.5 and 0.5 ml 'Eppendorff' microcentrifuge tubes (Fisher Scientific, Lougborough, Leics.)

Methods

i.Isolation of plasmid DNA

a. Plasmid isolation by modified Kado and Liu method (1981)

The method used was modified from that described by Kado and Liu (1981). Bacterial strains were grown from frozen stock isolates, or from plate cultures from the frozen stocks, overnight at 35^0 C in 10 ml of nutrient broth. 1.5 ml of culture was pelleted at 13,000 g for 4 min in a MSE Microcentaur microcentrifuge. The supernatant was poured off and the pelleted cells were lysed by agitating the pipette tip into the pellet and any residual broth followed by addition of 150 µl of lysing solution, then mixed to form a suspension. The suspension was heated at 65°C for 90 min in a waterbath. 150 µl of phenol/chloroform, produced by mixing equilibrated phenol, chloroform and isoamyl alcohol, in a ratio of 25:24:1 (Sambrook *et al* 1989), was added and an emulsion produced by vigorous shaking of the tubes. The emulsion was broken by centrifugation at 13,000 g for 3 min. 75 µl of the upper aqueous layer was removed to a fresh tube using a pipette tip with the end removed to reduce shearing of large plasmids and the possibility of disturbing the precipitate at the interface. 7.5 µl of loading buffer was added to each sample prior to immediate loading onto an agarose gel. A number of modifications were also tried. The ratio of phenol:chloroform was varied, and a second phenol/chloroform extraction step also used.

b. Plasmid isolation by Birnboim method (1983)

1.5 ml of overnight culture was pelleted by centrifuging at 13,000 g in a MSE Microcentaur microcentrifuge for 5 min. The supernatant was decanted and the pellet resuspended in 100 µl of lysozyme solution, and placed on ice for 5 min. 200 µl of

alkaline SDS was added and the tubes mixed by inverting . The tubes were placed on ice for 5 min. 150 µl of high salt solution was added and the tubes placed on ice for 15 min to form a 'curd-like' precipitate. The precipitate was pelleted by centrifuging at 13,000 g for 2 min. 350 µl of supernatant was removed and pipetted into a clean tube. 900 µl of ice cold ethanol was added and the tubes maintained at – 20° C in a freezer for 5 min. The tubes were centrifuged for 1 minute and the supernatant decanted. The pellet was dissolved in 100 µl of acetate MOPS buffer. Plasmid DNA was precipitated by the addition of 200 µl of ethanol and pelleted for 2 min at 13,000 g. The ethanol was removed and the DNA pellet resuspended in 40 µl of TE or TBE buffer. 10 µl was removed and added to 5 µl of loading buffer and loaded immediately into an agarose gel. The remaining 30 µl was frozen at -70 °C for future use.

c. Modified Birnboim method for isolation of *B. cepacia* plasmid DNA (Lennon and DiCicco 1991)

The method follows the Birnboim method to the precipitation with 900 µl of ethanol. At this point the pellet was resuspended with 100 µl of TE buffer rather than acetate MOPS buffer. An equal volume of phenol/chloroform was added and the tubes shaken vigorously to form an emulsion. The emulsion was broken by centrifuging at 13,000 g for 3 min. The upper aqueous layer was removed to a clean tube. 100 µl of acetate MOPS was added and the remainder of the method was as for the Birnboim method.

d. Isolation of plasmid DNA by commercial kits and the Rodriguez and Tait method (1983)

A number of commercial kits for plasmid isolation were also used. Qiagen plasmid mini kits and QiaFilter plasmid maxiprep kits were both tried with and without chloramphenicol amplification. These kits were used following manufacturers' instructions. Promega 'Wizard' kits were also used following manufacturers' instructions A number of modifications to the method were also used: 6 or 9 ml of culture were used for the initial bacterial pellet and/or a phenol/chloroform extraction was carried out on the lysate prior to mixing with the resin and loading on the column.

The Rodriguez and Tait method (1983) was also used, following the published protocol.

ii. Amplification of plasmid DNA with chloramphenicol (Sambrook *et al* 1989)

500 ml LB broths were pre-warmed to 37°C in a shaking incubator for 1 h. The 25 ml overnight cultures were added to the broths and the flasks were incubated at 37°C for 2.5 h shaking vigorously at 300 cycles min^{-1}. After the 2.5 h 2.5 ml of 34 mg ml^{-1} chloramphenicol was added to each of the flasks to give a final concentration of 170 μg ml^{-1}. The culture was then incubated for 15 h at 37°C maintaining the shaking at 300 cycles min^{-1}. The amplified culture was then used in the previously described Kado and Liu, modified Promega Wizard and Qiagen QiaFilter maxi methods(p.173). Amplification by growth in Terrific Broth was also used.

iii.Agarose gel plasmid DNA electrophoresis

0.21 g of agarose was weighed and placed into 30 ml of TBE buffer to give a 0.7% agarose gel. The gel was melted either in a microwave or over a Bunsen burner, mixed

well and allowed to cool before pouring. The cooled gel was poured into the chamber of a Gibco BRL Horizon 58 gel electrophoresis tank to give an approximate gel thickness of 5 mm. After allowing to cool and set for 1 h, TBE running buffer was added to the tank. 20 µl of samples with loading buffer was added to the wells of the agarose gel.

Electrophoresis was carried out at 4 V cm^{-1} for 90 to 120 min until the bromocresol tracking dye in the loading buffer had nearly reached the end of the gel. The gel was stained with ethidium bromide made to 0.5 µg ml^{-1} in distilled water for 15 min. Gels were then destained for 15 min under running tap water.

Gels were photographed over a short wave ultraviolet light (UV) source (UVP UV transilluminator). Polaroid 667 film was exposed through an UV filter , Kodak Wratten No.9 filter and F-11 aperture for 4 s in a Polaroid CU5 land camera fitted with 88-46 or 88-48 camera hoods. Film was developed at room temperature for 60 s.

iv. Restriction endonuclease digests of plasmid DNA

Restriction digests using the restriction endonucleases *Hind* III and *Eco*RI (Promega, Southampton, Hants) were set up in the supplied buffers under manufacturers' recommended conditions using plasmid DNA obtained from commercial plasmid DNA isolation kits outlined previously (P.174). λDNA (Promega, Southampton, Hants) digests were included as digestion controls. Digested samples were loaded onto 0.7% agarose gels and run at 3 V cm^{-1} for 1h. The gels were then stained and photographed as described above .

v. Isolation of plasmid DNA from preparative gels

. Samples were prepared by the modified Kado and Liu (1981) method using 3 ml pellets and double quantities of all buffers and solutions. Six sets of samples were prepared of each strain giving a final 1.2 ml volume of pooled supernatants. Preparative gels were cast in BioRad large sub-cells using 1.08 g of agarose in 180 ml of TBE yielding a 0.6 % agarose gel or in a BioRad sub-cell using 0.6 g of agarose in 100 ml of TBE to give a 0.6 % agarose gel. Gels were run at 4 V cm^{-1} and stained with ethidium bromide and the plasmid band was cut out. The gel slices were re-stained with ethidium bromide and checked under UV transillumination for presence of the DNA. The slice was macerated with an equal volume of TE buffer. The samples from were run on a standard 0.7 % agarose mini gel to check for the presence of plasmid DNA.

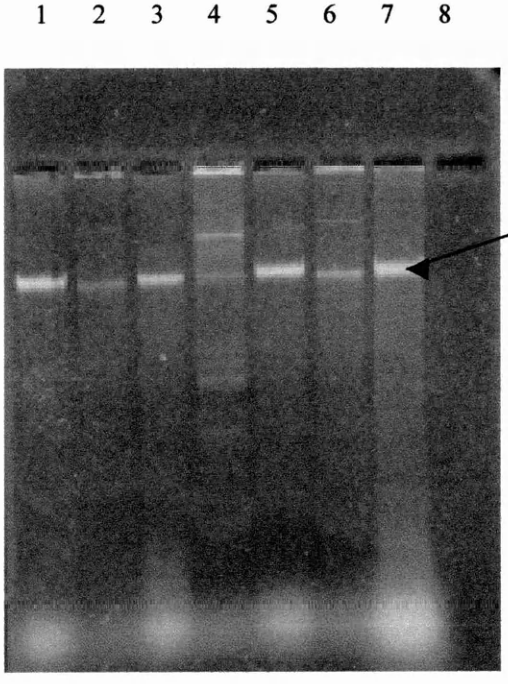
5.3 Results

5.3.1 Isolation of plasmid DNA

Plasmid DNA could not be isolated or detected in *B. cepacia* by the Rodriguez and Tait method, the Qiagen plasmid miniprep kit or the Promega Wizard miniprep kit though both the Qiagen and Wizard kits could detect the 2.7 kb plasmid pUC19 from *E. coli* DH5α. Extensive modification of the Wizard method (increasing pellet size and adding a phenol/chloroform extraction) detected a plasmid in *B. cepacia* C59 but not in other isolates .The method of Birnboim (1981) detected plasmid DNA in a number of *B. cepacia* isolates, but proved to be inconsistent in subsequent repeats. The modified method described by Lennon and DeCicco (1991) failed to detect plasmids in the *B. cepacia* strains tested .

The modified Kado and Liu method (1981) detected plasmids reasonably consistently in a range of *B. cepacia* strainss (Plate 5.1). On the basis of this the Kado and Liu method was chosen for the subsequent detection of plasmid DNA in all *B. cepacia* strainss and for their size estimation. A further example of typical results obtained is shown in Plates 5.2. In each of the gels fragments of chromosomal DNA can be seen, running approximately equivalent to 10 kb, as marked on the gels (Burton, personal communication 1994).

Plate 5.1 Agarose gel electrophoresis characterisation of plasmids isolated by the modified Kado and Liu method (1981) and *E. coli* V517 standard reference plasmids



Lane

1 *B. cepacia* C51

2 *B. cepacia* C5

3 *B. cepacia* C95

4 *E. coli* V517 marker strain

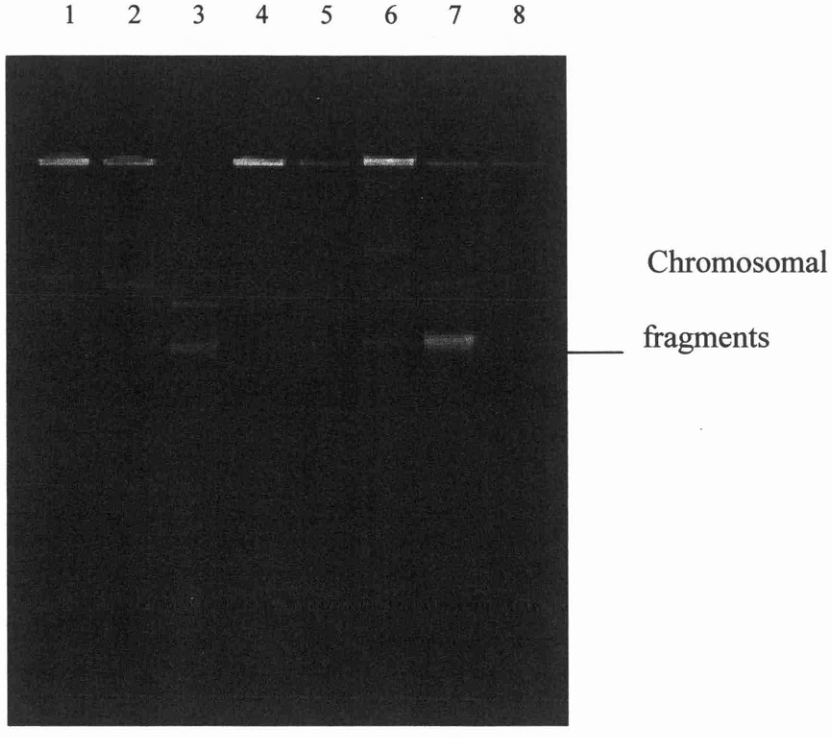
5 *B. cepacia* C11

6 *B. cepacia* C1

7 *Ps. aeruginosa* PAO1/pQM1/R300B

8 Blank

Plate 5.2 Agarose gel electrophoresis characterisation of plasmids isolated by the modified Kado and Liu method (1981) and *E. coli* V517 standard reference plasmids



Lane

1 *E. coli* V517 marker strain

2 *B. cepacia* PW7

3 *B. cepacia* PW6

4 *B. cepacia* PW5

5 *B. cepacia* PW4

6 *B. cepacia* PW3

7 *B. cepacia* PW2

8 *B. cepacia* PW8

5.3.2 Estimation of plasmid size and distribution of plasmids

Plasmid size was estimated from agarose gels by regression analysis of $\log_{10}$ molecular size (kb) against $\log_{10}$ mobility or distance migrated (mm) using *E. coli* V517 or 39R plasmids as standards. The sizes of 8 plasmids in V517 and the 4 in 39R have been well characterised and used as size markers (Macrina *et al* 1979, Threlfall *et al* 1986). The distance migrated was measured with a calliper or rule as the distance from the origin (front of well) to the leading edge of the band on the Polaroid photograph. The data was analysed by MINITAB for Windows .

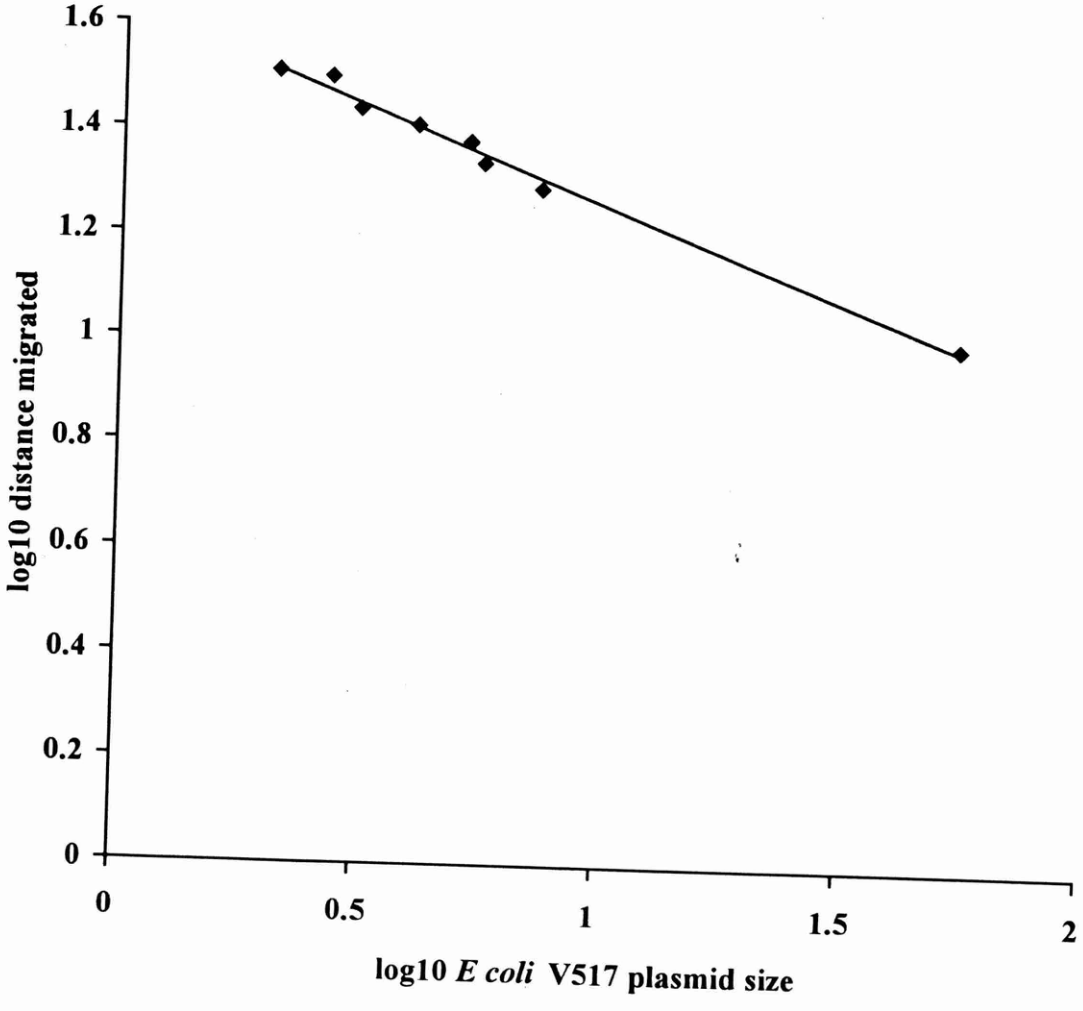
Example calculation

Using Plate 5.2 with V517 as standard (lane 1), the plasmid sizes and distances migrated:

Distance migrated (mm)	Plasmid size (kb)
10	55.1
19	7.4
22	5.6
24	5.2
26	4.1
28	3.1
32	2.7
33	2.1

The values for both distance travelled and plasmid size were $\log_{10}$ transformed, and $\log_{10}$ molecular size was plotted against $\log_{10}$ distance travelled (Figure 5.1), with simple linear regression analysis used to give the regression equation.

Figure 5.1 **Standard plot of $\log_{10}$ molecular size against $\log_{10}$ distance travelled for *E. coli* V517 from Plate 5.2**



The regression equation obtained :

$y = c\text{-}mx$

$y = 1.64 - 0.374\ x$

Where $y = \log_{10}$ distance migrated

$x = \log_{10}$ plasmid size

As the distance migrated by the plasmids can be obtained from the gel the value for y can be substituted for the distance migrated by a plasmid of unknown size.

Therefore for the plasmid in PW6, the distance migrated = 12 mm

$\log_{10} 12 = 1.64 - 0.374\ x$

Where $x = \log_{10}$ plasmid size

$1.08 = 1.64 - 0.374\ x$

$$\frac{1.08-1.64}{-0.374} = x$$

$1.49 = x$

antilog 1.49 = plasmid size (kb)

31.4 kb = plasmid size in PW6

The plot was well predicted with a good line of fit shown by the coefficient of correlation and Durbin Watson values. The coefficient of correlation (squared correlation coefficient value), R^2 was 98.7%. The method was subsequently used to estimate the size of plasmids from agarose gels using V517 and 39R size standards,

with an individual regression equation obtained for the plasmid standards in each gel The regression analysis gave both good consistency and prediction for line of fit. Consistently high values were obtained for R^2 . From 14 gels a mean R^2 value of 98.73% was found, with values ranging from 97.1% to 99.8%.

Some difficulty arose with larger plasmids (>150 kb) as the 251 kb plasmid pQM1 was not detected reliably by agarose gel electrophoresis and so could not be used as a size standard. Therefore the 151 kb plasmid of 39R was used as the largest standard and it was necessary to extrapolate the regression equation beyond the values of the standard to estimate the sizes of the larger plasmids.

Plasmids detected in the *B. cepacia* plasmids and their estimated sizes are given in Table 5.2. The data shown here represents mean values obtained for each plasmid for a minimum of two repeated gels, though with some strains up to eight repeat experiments were conducted. Figure 5.2 shows the frequency distribution of plasmids found in the *B. cepacia* strainss investigated in this study.

Table 5.2 Size and distribution of plasmids in tested *B. cepacia* isolates

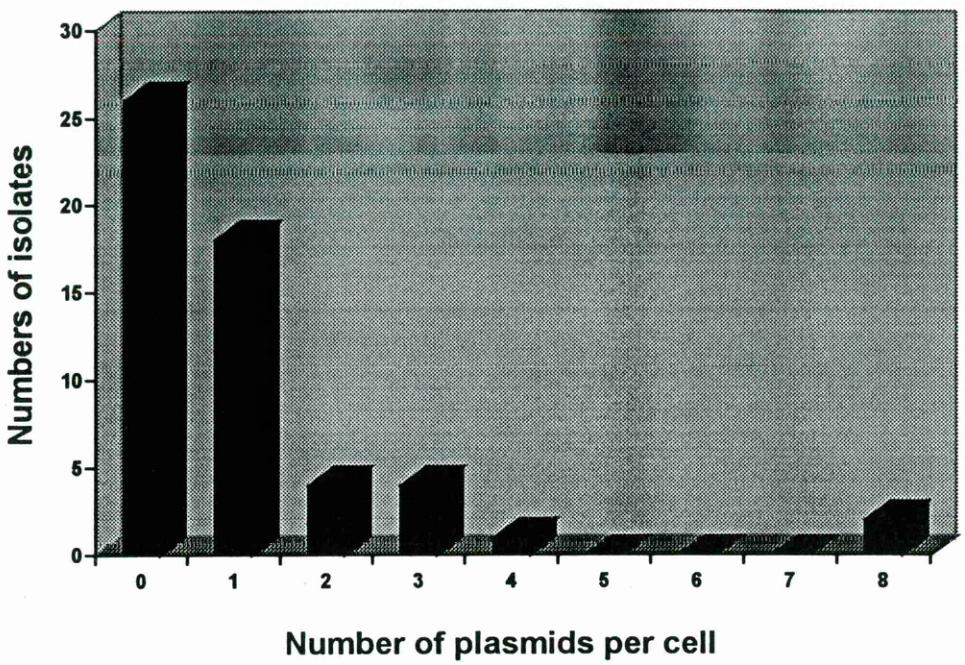
Isolate	Plasmid sizes (kb)
C1	134
C5	134
C11	134
C23	139, 24, 13
C49	2.1, 1.7
C51	ND
C59	20
C79	90
C81	40
C93	ND
C95	ND
C96	109, 2.1, 1.7
C116	144, 29.3, 2.1,1.7
C187	51, 4.4, 4.0
C190	ND
C205	ND
J543	90
J478	152
A548	ND
A562	70, 4.5
C1858	ND
C1860	185, 3.4
J2552	ND
D2	41
D3	ND
D4	36
D5	36
D6	ND
NCPPB 2993	224
NCPPB 3480	200, 177
NCIMB 9085	58
NCIMB 9087	ND
NCIMB 9088	38
NCIMB 9092	ND
PW1	ND
PW2	132, 51, 31, 16, 9, 8, 3.6, 1.1
PW3	93
PW4	31
PW5	ND
PW6	31

Table 5.2 (Continued)

PW7	132, 51, 45, 11, 7, 6.2, 3.6, 2.5
PW8	ND
PW9	>250
PW10	16, 12, 7.1
PW11	ND
PW12	ND
PW13	ND
PW14	ND
PW15	ND
PW16	ND
PW17	ND
PW18	ND
PW19	ND
PW20	ND
PW21	ND
B. gladioli NCPPB 2478	116

ND = no plasmid(s) detected

Figure 5.2 Frequency of plasmids in *B. cepacia*



5.3.3 Isolation of plasmid DNA for transformation and plasmid mapping studies.

As insufficient plasmid DNA was isolated for transformation or mapping of plasmids by the mini-prep and commercial methods used, it was decided to use commercial maxi prep methods. Use of the Qiagen QiaFilter maxiprep kit detected small amounts plasmid DNA in NCPPB 2993 and C79. Digestion with *Eco*RI or *Hin*d III failed to yield any fragments. This was also found using plasmid DNA from the Kado and Liu method. The use of preparative gels also failed to yield plasmid DNA.

5.3.4 Amplification of plasmid DNA by growth in Terrific broth and by chloramphenicol amplification.

Growth in Terrific broth was unsuccessful in increasing plasmid yield. Growing overnight cultures in Terrific broth increased overall growth and the amount of DNA leading to smearing and a large chromosomal fragment band The use of chloramphenicol amplification failed to increase plasmid DNA by Kado and Liu (1981), QiaFilter maxiprep or modified Promega Wizard miniprep methods. In both the QiaFilter and Wizard methods, the plasmid yield appeared to decrease.

5.4 Discussion

5.4.1 Characterisation of plasmid DNA

Generally the methods used in this study were poor at detecting and characterising plasmid DNA in *B. cepacia* . The Qiagen miniprep, Rodriguez and Tait (1983) or Promega Wizard methods failed to detect plasmid DNA. The Birnboim method was reasonably good in detecting smaller plasmid but was inconsistent in detecting larger plasmids. The modified method of Lennon and DiCicco (1991), developed for *B. cepacia* plasmids, was unsuccessful in this study. Modification of the Promega Wizard miniprep by increasing pellet size and using a phenol/chloroform extraction was only able to detect a small 20 kb plasmid from C59. The Kado and Liu (1981) method appeared to be the best method overall, though this also failed to yield sufficient quantities of purified DNA for future work, though was a good method for plasmid screening.

A number of groups have had difficulty in isolating plasmids in *B. cepacia*. Both Williams *et al* (1979) and Hirai *et al* (1982) found difficulty in isolating antibiotic resistance plasmids from *B. cepacia.* Williams *et al* (1979) suggested that nucleases present in the extraction method might cause problems in obtaining consistent plasmid profiles. Lennon and DiCicco (1991) found difficulty in obtaining consistent plasmid profiles with either the Kado and Liu or Birnboim methods, with consistency only achieved by the addition of a phenol/chloroform extraction step to the Birnboim method though in this study the Kado and Liu method was found to be more consistent. Other groups have also found success with versions of the Kado and Liu

method (Bhat *et al* 1995). Large degradative plasmids have also been detected in *Pseudomonas* sp. by this method (Cork and Khahlil 1995). The failure to isolate plasmid DNA from *B. cepacia* by these methods was one of the main factors considered when trying to develop the use of commercial kits to obtain a high yield of DNA, though these were with hindsight even less suitable for use with *B. cepacia.*

Factors that may contribute to the poor isolation of *B. cepacia* plasmid DNA are a low plasmid copy number, the large size of the plasmids and the nature of *B. cepacia* itself, particularly its' cell wall structure. The cell wall structure may not be broken by 'milder' lysing methods employed in cleared lysate methods such as the Rodriguez and Tait method, so no plasmids are released. Large plasmids may be sheared by conventional methods. The low copy number may mean that there is too little DNA to be detected by agarose gel electrophoresis.

Both large size and low copy number could lead to problems with commercial kits. These methods are developed mainly for use with small high copy number plasmids such as vector plasmids like pGEM. Large plasmids, often in excess of 100 kb, are found in *B. cepacia*, and even with a raised elution temperature cannot be isolated by these methods. The methods are also usually employed with plasmids in *E. coli*. As mentioned previously, it is significant that the only plasmid isolated in *B. cepacia* by commercial methods, was the relatively small 20 kb plasmid in C59 by a highly modified Promega Wizard method.

It would appear that the stronger lysis of the Kado and Liu method is more successful in breaking the *B. cepacia* cell wall and that the rather crude phenol/chloroform extraction is the most successful method to isolate *B. cepacia* plasmids. On the basis of this it was chosen to estimate the size of plasmids and their distribution in *B. cepacia.*

5.4.2 Sizing of *B. cepacia* plasmids

The use of regression analysis of $\log_{10}$ molecular size against $\log_{10}$ of the distance migrated gave good consistent results, with well predicted lines of fit with a mean R^2 value of 98.7%. Log_{10} of the distance travelled rather than $\log_{10}$ of the relative mobility used by Meyers *et al* (1976) or Rochelle *et al* (1985) was used in this study. A simple linear regression was used in this study rather than multiple regression used by Rochelle *et al* (1985) who obtained more accurate and consistent values using multiple regression of $\log_{10}$ molecular weight against $\log_{10}$ of the relative mobility and reciprocal square root of the mobility. This gave a mean R^2 value of 98.6% compared with 97.5% for a single linear regression. In this study the mean R^2 value of 98.73% showed a good linear relationship with little variance, the high R^2 value indicating no real requirement for multiple regression. Rochelle *et al* (1985) also stated that the single linear regression underestimated the sizes of large plasmids. The only benchmark to which the estimated sizes can be compared in this study is the large plasmid in the species type strain NCPPB 2993/ATCC 25416, that has been reported as being 212 kb (Lennon and DiCicco 1991) or 220 kb (Rodley *et al* 1995) by restriction mapping. In this study plasmid size was estimated at 224 kb.

The main problems came from estimating the size of larger plasmids. The failure to detect the 251 kb plasmid pQM1 with any consistency meant the largest available standard was the 151 kb plasmid of *E. coli* 39R. Therefore to estimate the size of the larger plasmids the regression equation was extrapolated to values outside the standards. Though this is not regarded as good statistical practice, the high correlation of the line, with a mean R^2 value of 98.75% over the size range of 2.1 kb to 151 kb, and that the larger plasmids were only slightly beyond the standard size range were taken into account before making the decision to extrapolate the regression equation to estimate the larger sizes. The relatively good agreement between published estimates of the large plasmid in NCPPB 2993 and those found here suggests this was reasonably successful Good reproducibility between gels was also achieved, and also between V517 and 39R as standards. This is best illustrated by size estimation of the large plasmid in NCPPB 3480. The plasmid was estimated to be 177 kb with 39R as the standard, and 178 kb with V517 as the standard. However, these sizes can only really be regarded as a rough estimation of size. A number of factors such as the relatively small difference in distances migrated by plasmid molecules on the mini gels used, the potential presence of cccDNA and ccDNA forms of the plasmid may lead to large inaccuracies of measurement. It is almost certainly the case that restriction digestion of the large plasmids in *B. cepacia* would be far more accurate and consistent in size estimation, though the failure to isolate sufficient pure plasmid DNA meant this method could not be used.

5.4.3 Distribution and sizes of plasmids

In total 28 of 54 *B. cepacia* strains were found to contain plasmids (52 %) (Table 5.2). 17 of the 26 *B. cepacia* CF strains were found to contain plasmids (65 %). This is considerably more than the 23 % of *B. cepacia* CF strains (11 of 48) found to contain plasmids by McKevitt and Woods (1984), but somewhat lower than the 92 % of clinical and environmental strains containing plasmids reported by Gonzalez and Vidaver (1979) or the 82 % of clinical and environmental strains containing plasmids reported by Lennon and DeCicco (1991).

Only 11 of 28 environmental isolates were found to contain plasmids (39 %). It was notable that 5 of 7 (71 %) of isolates obtained from industrialised regions of South Wales contained plasmids compared with only 2 of 13 (15%) of isolates from the Brecon Beacons national park. Two isolates from water downstream of a former colliery (PW2) or from soil near to a coking works (PW7) both showed multiple plasmids. Both isolates contained 8 plasmids, with both containing 132 kb and 51 kb plasmids. It is possible that plasmids from these sites may encode for degradative properties that would give an advantage in polluted sites, whereas in clean sites there is no requirement for such properties and hence no plasmids.

Few plasmids of greater than 200 kb were found in this study, whereas Lennon and DeCicco (1991) found 20 of 37 isolates contained plasmids of 208, 212 or 222 kb, though in this study 13 isolates were found to contain plasmids larger than 100 kb. PW9 (and possibly C116) was found to contain a megaplasmid in excess of 250 kb. McKevitt and Woods (1984) found plasmids in the size range of approximately 70 to

150 kb in *B. cepacia* CF isolates. Similar sizes were found in this study (Table 5.2), though a number of smaller plasmids (between 2 to 40 kb) were also found.

It is notable that *B. cepacia* isolates from CF siblings were not only of the same ribotype (Chapter 8 p.258) but had plasmids of identical or of similar sizes. Isolates C5 and C11 from siblings were found to contain a 134 kb plasmid. C1 was found to contain a plasmid of 134 kb, whilst C23 isolated from a sibling contained a 139 kb plasmid. It is possible that these plasmids are the same, but differences in sizes were estimated as being different from different gels. Plasmids of a similar size (132 kb) were also found in the environmental isolates PW2 and PW7. To assess the relatedness of these plasmids, restriction mapping could be used, provided sufficient DNA could be isolated.

5.4.4 Isolation of *B. cepacia* plasmid DNA for restriction mapping and transformation studies.

Many of the problems associated with small scale isolation or detection of plasmid DNA also apply to the larger scale isolation of plasmid DNA. The QiaFilter maxiprep kit was able to isolate the large 224 kb plasmid in the species type strain. However this was accompanied by a considerable amount of chromosomal DNA. The yield of DNA was also poor, as shown by the failure to obtain a digest pattern with either *Eco*RI or *Hin*d III.

The attempts at using the Kado and Liu method with preparative gels also resulted in failure to obtain any significant quantities of plasmid DNA. Attempts to increase

plasmid DNA by growth in Terrific broth or by chloramphenicol amplification were made (Sambrook *et al* 1989), though both methods proved unsuccessful. The lack of success of the chloramphenicol amplification may be largely explained by the recent discovery of an active chloramphenicol efflux mechanism preventing accumulation of the drug within *B. cepacia* cells (Burns *et al* 1996b).

From this study it would appear that none of the methods used are particularly suitable for isolation of plasmid DNA for use in transformation or restriction mapping of the plasmids. Caesium chloride equilibrium gradient centrifugation, as used by Haak *et al* (1995) to isolate the degradative plasmid pBAH1, may be the only viable method for isolation of *B. cepacia* plasmid DNA.

5.4.5 Improvements, alternatives and future work

As outlined in the previous sections, the detection of plasmid DNA in *B. cepacia* has proved difficult. Although a number of methods have been applied in this study, this is by no means an exhaustive list of methods. Cork and Khalil (1995) described a number of methods for isolating megaplasmid DNA (250 kb) from *Pseudomonas* sp. Cork and Khalil (1995) found that boiling methods such as those of Holmes and Quigley (1981)cleared lysate methods or polyethylene glycol precipitation all failed. The method of Wheatcroft and Williams(1982), using SDS and sodium hydroxide lysis then direct running of the sheared lysate on an electrophoresis gel, and the use of, SDS lysis followed by a phenol extraction were both successful , though the method of Kado and Liu produced better results and was more convenient to perform. A more gentle lysis method with lysozyme, EDTA, glucose, sucrose and tris followed by SDS, then two

precipitations of the DNA with sodium acetate and isopropanol was found to be the most successful and reproducible method. Additionally the Hansen and Olsen method (1978) could be tried, as this has proved successful in isolation of large plasmids in *B. cepacia* (Xia *et al* 1996).

The other main improvement could be made in plasmid size estimation. The use of simple linear regression of $\log_{10}$ molecular size against $\log_{10}$ distance migrated would appear to be a good, consistent method of size estimation. The main problem being the larger plasmid sizes. Improved isolation could allow sizing by restriction digest analysis. Alternatively more consistency in use of a large plasmid standard may be achieved by an alternative method. The 224 kb plasmid in the species type strain may be a candidate for such a standard.

Chapter 6 Plasmid curing and its effect on phenotype

6.1 Introduction

Curing is the spontaneous loss of a plasmid-borne characteristic, either through loss of a complete plasmid or through dissociation of plasmid genes. Curing may be promoted through the use of curing agents that affect DNA replication such as ethidium bromide, acridine orange or ultraviolet light (Day 1982). Curing can be used in the determination of phenotypic properties of plasmids. The loss of a phenotype correlating with the loss of the plasmid indicates it may encode for that property.

Curing of plasmids encoding for antibiotic resistance may be achieved by culture on non-selective media. This was used as the basis of the curing method in this study, where *B. cepacia* isolates were serially transferred into fresh nutrient broth for 25 transfers. Loss of antibiotic resistance was determined by failure to grow on media containing antibiotics at levels lower than the MIC level. The loss or partial loss of plasmid DNA was confirmed by the modified Kado and Liu method (1981) described in Chapter 5, followed by gel electrophoresis.

6.2 Materials and methods

Materials

i.Strains

B. cepacia strains as described in Chapter 2

ii. Media

10 ml aliquots of nutrient broth (Oxoid, Unipath Ltd., Basingstoke, Hants.)

20 ml aliquots of molten DST agar, maintained at 45 to 50 °C (Oxoid, Unipath Ltd., Basingstoke, Hants.)

Nutrient agar plates (Oxoid, Unipath Ltd., Basingstoke, Hants.)

iii. Solutions

Sterile 0.85 % saline

Stock solutions of ceftazidime, gentamicin and piperacillin at 5 mg ml^{-1}, prepared as in Chapter 4 (Sigma Chemical Co.,Poole, Dorset)

iv. Disposable consumables

Sterile wooded toothpicks

Petri dishes.

In addition the materials for plasmid isolation by the Kado and Liu (1981) method as outlined in Chapter 5 (p.173) were used.

Methods

ii.Plasmid curing

Curing was carried out by the serial transfer of a loopfull of *B. cepacia* static broth culture grown at 35 °C as previously described (Chapter 2 p.97), from the frozen stock of wild type, into fresh NB daily for 25 days. After the 25th transfer, dilutions of the culture from 10^{-3} to 10^{-5} in sterile saline were produced and plated onto NA plates and

incubated to give discrete colonies of *B. cepacia*, ideally between 100 to 200 colonies. 100 or 50 colonies were picked from the plates to give a good representative sample, and then replica plated onto DST agar plates containing sub-MIC levels of gentamicin, ceftazidime and piperacillin to determine any loss of antibiotic resistance. These plates were produced by adding stock solutions of the antibiotics to 20 ml of molten DST agar, pouring and allowing to set and dry. The final antibiotic concentration was approximately half of the MIC level for that strain. The levels used were determined from the MIC levels described in Chapter 4 for each of the strains used. These ranged from 1 to 5 μg ml^{-1} for ceftazidime, 5 to 20 μg ml^{-1} for piperacillin and 10 to 50 μg ml^{-1} for gentamicin. A template was used to place 25 inoculum sites onto a single plate and to interpret their position after incubation. Following inoculation onto the 3 antibiotic plates, the picked colony was inoculated onto a nutrient agar plate, using the template as above, The nutrient agar acted to determine viability for each colony tested.

The inoculated plates were incubated at 35°C for 48 h. After incubation growth on each plate was recorded. Where a colony failed to grow on the antibiotic plate but grew on the nutrient agar, it was said to have lost or been 'cured' of a resistance phenotype to that antibiotic. The number of colonies of each isolate failing to grow was recorded and percentage of colonies cured of the phenotype calculated. A selection of 'cured' colonies was sub-cultured from the control plate for investigation of plasmid loss.

ii.Investigation of plasmid loss in 'cured' strains

The loss of plasmid DNA was investigated by the modified Kado and Liu method, followed by agarose gel electrophoresis as described in Chapter 5 (p.175). 'Cured'

strains were run alongside the original *wild type* stock isolates, previously confirmed as containing plasmids.

6.3 Results

6.3.1 Loss of antibiotic resistance by curing

The numbers of colonies and percentage of each isolate found to have lost antibiotic resistance after curing by serial transfer is shown in Table 6.1:

Table 6.1 Numbers and percentage of *B. cepacia* strains cured of antibiotic resistance by serial transfer

Strain	Growth on nutrient agar to confirm viability	Number of colonies failing to grow on sub-MIC antibiotics (% cured of antibiotic resistance)		
		Ceftazidime	Gentamicin	Piperacillin
C5	96/100	NR	0 (**0**)	0 (**0**)
C23	91/93	NR	0 (**0**)	3 (**3**)
C1860	49/50	0 (**0**)	32 (**66**)	4 (**8**)
A562	98/100	0 (**0**)	4 (**4**)	0 (**0**)
NCIMB 9085	100/100	0 (**0**)	0 (**0**)	0 (**0**)
J543	100/100	0 (**0**)	0 (**0**)	0 (**0**)
C190	50/50	9 (**18**)	0 (**0**)	8 (**16**)
NCPPB 3480	49/50	13 (**26**)	0 (**0**)	8 (**16**)
C116	100/100	6 (**6**)	0 (**0**)	4 (**4**)
NCPPB 2993	49/50	3 (**6**)	1 (**2**)	1 (**2**)
C187	49/50	4 (**8**)	0 (**0**)	4 (**8**)
J478	47/50	10 (**21**)	0 (**0**)	7 (**15**)

NR = No result obtained

6.3.2 Investigation of plasmid loss

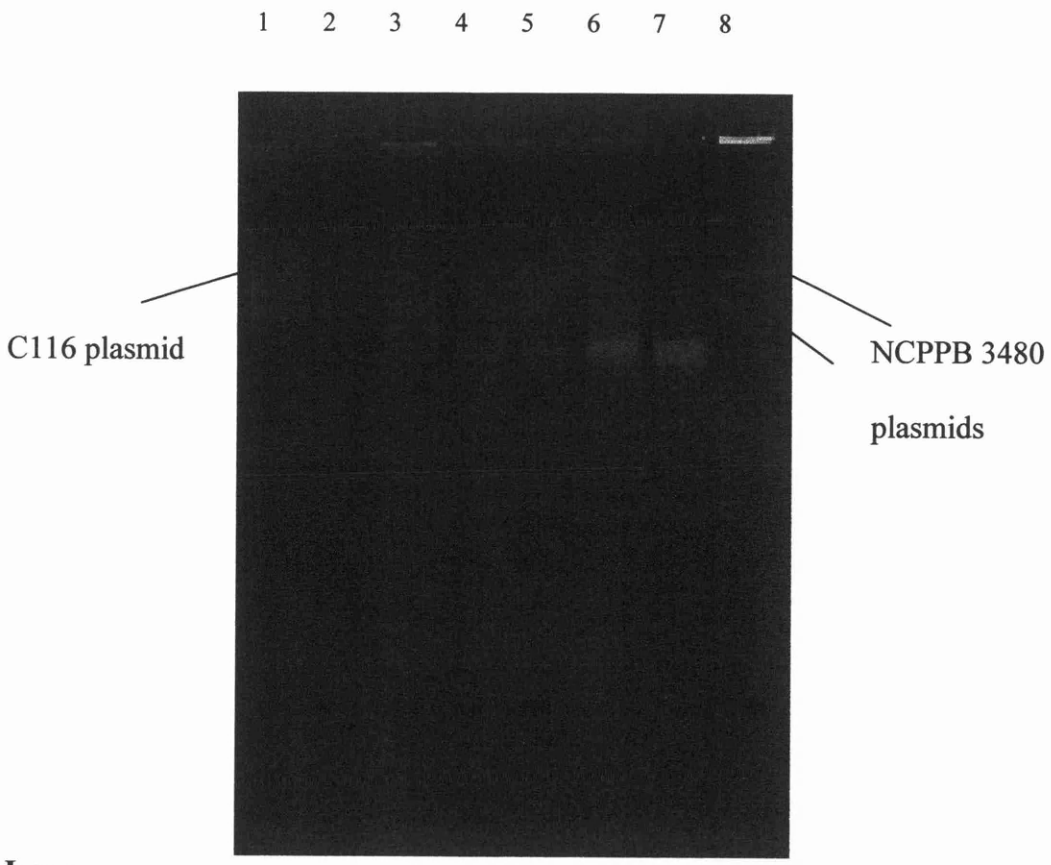
Using the modified Kado and Liu method, plasmid loss was seen in 5 of the 9 strains in which there was evidence of loss of antibiotic resistance by curing. The plasmids lost and the loss of resistance associated with the colony are summarised in Table 6.2

Table 6.2 Summary of plasmid loss and resistance loss in cured *B. cepacia* strains

Isolate	Plasmid loss and loss of resistance
C5	None
C23	None
C1860	Loss of 185 kb plasmid in 3 /3 (3 out of 3) colonies tested with loss of gentamicin resistance
A562	None
C190	None
NCPPB 3480	200 kb plasmid lost in 1/3 colonies tested with loss of ceftazidime resistance and 1/ 2 with loss of piperacillin resistance. 177 kb and 200 kb plasmids lost in 1/2 colonies tested with loss of both piperacillin and ceftazidime resistance. Change of position (decreased size) of both plasmids in 1/3 colonies with loss of ceftazidime resistance
C116	29 kb plasmid lost in 1/1 colonies tested with loss of piperacillin resistance, and 1/1 colonies with loss of ceftazidime resistance. No loss found in colonies losing both ceftazidime and piperacillin resistance
NCPPB 2993	224 kb plasmid lost in 1/1 colonies with loss of ceftazidime resistance and 1/1 colonies with loss of gentamicin resistance
C187	None
J 478	152 kb plasmid lost in 2 /2 colonies with loss of piperacillin resistance and 1/1 colony with loss of piperacillin and ceftazidime resistance

Polaroid photographs showing the loss of plasmids are shown in plates 6.1 to 6.4

Plate 6.1 Plasmid presence in cured and *wild-type B. cepacia* strains



Lane

1 Cured *B. cepacia* C116 -loss of piperacillin resistance

2 Cured *B. cepacia* C116 –loss of ceftazidime resistance

3 *B. cepacia* C116- original stock isolate 29 kb plasmid

4 Cured *B. cepacia* NCPPB 3480-loss of piperacillin resistance

5 Cured *B. cepacia* NCPPB 3480-loss of piperacillin and ceftazidime resistance

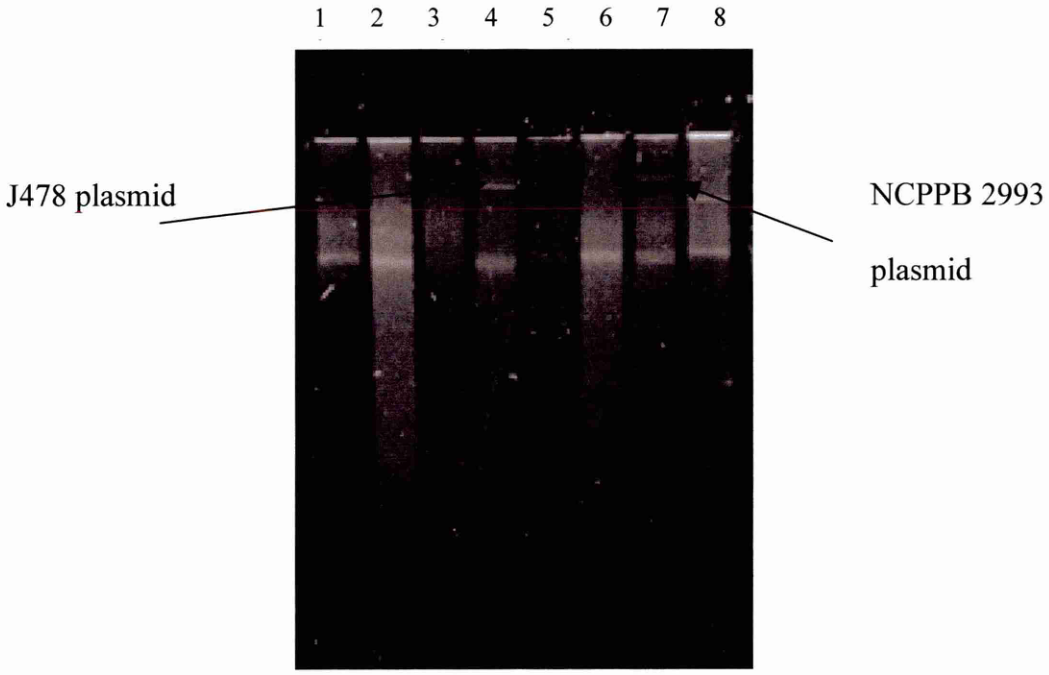
6 Cured *B. cepacia* NCPPB 3480-loss of ceftazidime resistance

7 *B. cepacia* NCPPB 3480 original stock isolate- 200 and 177 kb plasmids

8 *E. coli* V517 plasmid strain marker

In Plate 6.1 the 29 kb plasmid of C116 can be faintly seen in lane 3. In the cured strains in lanes 1 and 2 there is no evidence of the plasmid, indicating probable loss during curing. 2 plasmids of 200 and 177 kb can be seen in NCPPB 3480 in lane 7 but faintly. There is evidence of bands in lanes 4, 5 and 6 but the results are too faint to interpret. The NCPPB 3480 cure strains are repeated in Plate 6.4.

Plate 6.2 Plasmid presence in cured and *wild-type B. cepacia* strains



Lane

1 Cured *B. cepacia* J478- Loss of piperacillin and ceftazidime resistance

2 Cured *B. cepacia* J478- Loss of piperacillin resistance

3 Cured *B. cepacia* J478- Loss of piperacillin resistance

4 *B. cepacia* J478 *wt*stock isolate- 152 kb plasmid

5 Cured *B. cepacia* NCPPB 2993- loss of gentamicin resistance

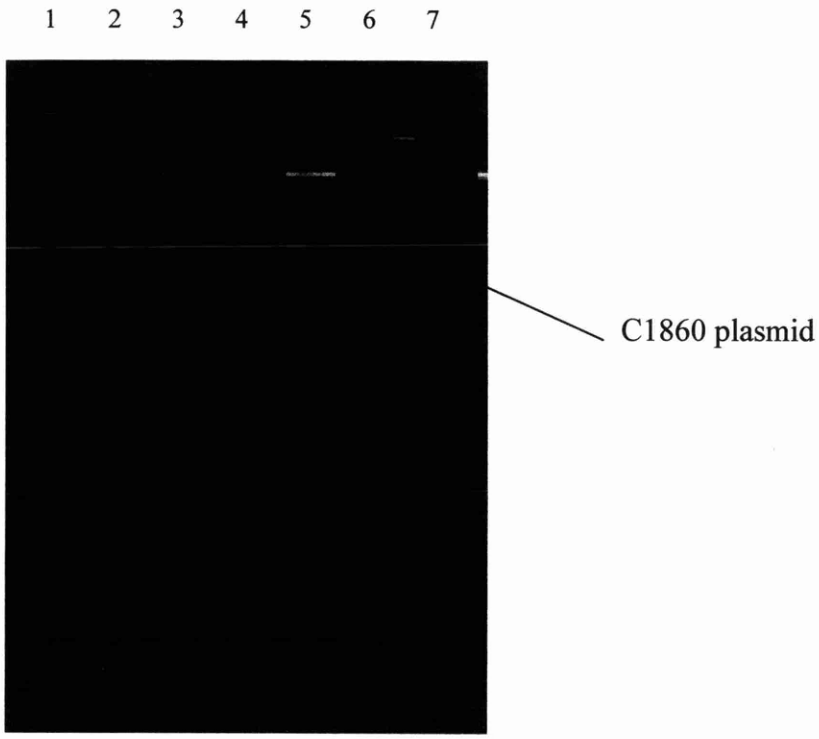
6 Cured *B. cepacia* NCPPB 2993- loss of ceftazidime resistance

7 *B. cepacia* NCPPB 2993 *wt* stock isolate - 224 kb plasmid

8 *E. coli* V517 plasmid marker strain.

B. cepacia J478 contains a single large plasmid of 152 kb that can be seen clearly in lane 4. The cured J478 colonies in lanes 1, 2 and 3 show no evidence of the plasmid indicating loss of the plasmid. *B. cepacia* NCPPB 2993 contains a single plasmid of 224 kb, as can be clearly seen in lane 7. There is no evidence of plasmids in the cured NCPPB 2993 in lanes 5 and 6, again suggesting loss of the plasmid.

Plate 6.3 Plasmid presence in cured and *wild-type B. cepacia* strains



Lane

1&2 Blank

3 Cured *B. cepacia* C1860- loss of gentamicin resistance

4 Cured *B. cepacia* C1860- loss of gentamicin resistance

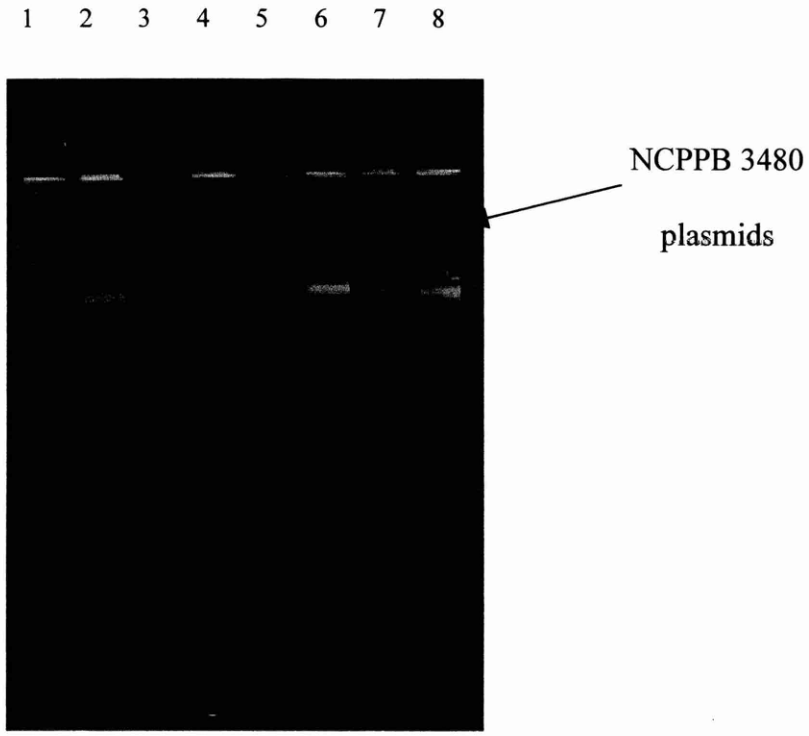
5 Cured *B. cepacia* C1860- loss of gentamicin resistance

6 *B. cepacia* C1860 *wt* stock isolate- 185 kb plasmid

7 Blank

B. cepacia C1860 , lane 6, shows a single large plasmid. The cured C1860 show no evidence of a band, indicating loss of the plasmid.

Plate 6.4 Plasmid presence in cured and *wild-type B. cepacia* strains



Lane

1 *E. coli* V517 reference strain

2 Cured *B. cepacia* NCPPB 3480- loss of resistance to ceftazidime

3 Cured *B. cepacia* NCPPB 3480- loss of resistance to piperacillin and ceftazidime

4 Cured *B. cepacia* NCPPB 3480- loss of resistance to piperacillin

5 Cured *B. cepacia* NCPPB 3480- loss of resistance to ceftazidime

6 Cured *B. cepacia* NCPPB 3480- loss of resistance to ceftazidime

7 *B. cepacia* NCPPB 3480 *wt* stock isolate- 200 and 177 kb plasmids

8 Cured *B. cepacia* NCPPB 3480- loss of resistance to piperacillin

B. cepacia NCPPB 3480, lane 7 shows 2 plasmids of 200 and 177 kb. The strains of NCPPB 3480 in lanes 8 and 6 show the same plasmid arrangement indicating curing has not occured. The cured strains in lanes 4 and 5 show the loss of the larger 200 kb plasmid but retain the smaller plasmid. In lane 2 a change of size of the 200 kb plasmidseems to have occured. The cured isolate containing a plasmid that appears slightly smaller than 200 kb,.though this is difficult to confirm as the difference in mobility on a mini-gel is small.

6.4 Discussion

Evidence of loss of an antibiotic resistance phenotype occurred in 10 of 12 strains cured by serial transfer. Plasmid loss was subsequently confirmed by gel electrophoresis in 5 of the 10 strains. Loss of resistance to piperacillin was most common, occurring in 7 strains, loss of ceftazidime resistance occurred in 6 strains and loss of gentamicin resistance occurred in 4 strains. The levels of gentamicin resistance loss were low, with the exeption of C1860, with levels of less than 4%. The levels of curing of ceftazidime and piperacillin resistance were generally higher ranging from 6 to 26% for ceftazidime and 2 to 16% for piperacillin. In a number of cases the resistance to both piperacillin and ceftazidime were lost by the same clone. The rates of curing for piperacillin and ceftazidime were also similar in a number of strains. For instance C190 had a curing rate of 16% for piperacillin and 18% for ceftazidime, C187 had a rate of 8% for both.

The above evidence suggests that plasmids bearing genes that encode for antibiotic resistance may occur in *B. cepacia*. Wilkinson and Pitt (1995b) stated there had been little exploration of the role of plasmids in antibiotic resistance in *B. cepacia*. As described in Chapter 1 only two cases of antibiotic resistance plasmids have been reported in *B. cepacia*; a 49kb streptomycin and mercury resistance plasmid (Hirai *et al* 1982), and 4.4kb tetracycline and 8 kb ampicillin resistance plasmids in strain 4G9 (Williams *et al* 1979). The curing levels for the β-lactam drugs piperacillin and ceftazidime would suggest some plasmid mediated resistance to these drugs. Such resistance is likely to be mediated by a β-lactamase enzyme. Plasmid encoded β-

lactamases have been widely reported in *Ps. aeruginosa* (Prince 1986), but not in *B. cepacia,* though there is strong evidence of chromosomally encoded enzymes (Baxter and Lambert 1995).

β-lactamases can be detected and typed by a number of biochemical tests such as starch hydrolysis (British Society for Antimicrobial Chemotherapy 1991). Inducible β-lactamases that are only induced by the stimulus of *β*-lactam drugs, such as those in *B. cepacia,* can be detected by the use of two antibiotics, one a strong inducer, the other a weak inducer in disc approximation tests or by incorporating a strong inducer into agar in a disc diffusion test using a disc of the weak inducer. An inducible enzyme would give an inhibition zone with the strong inducer, but smaller or no zone would occur without the inducer (British Society for Antimicrobial Chemotherapy 1991). Comparison of β-lactamase production between cured and uncured *B. cepacia* strains could be used to confirm the presence of a β-lactamase encoded by the cured plasmid, though the presence of chromosomally encoded plasmids may adversely affect the results.

Transfer of antibiotic resistance either by transformation or conjugation could be used to confirm the presence of a plasmid encoding for antibiotic resistance. However the failure of methods used in Chapter 5 to obtain plasmid DNA for transformation prevents its' use in this context. Although conjugal transfer of plasmids may be achieved (see Chapter 7), this does not rule out the possible transfer of chromosomal elements. The transfer of a plasmid to a β-lactamase free strain could be used to allow analysis of possible plasmid encoded β-lactamases.

The levels of curing of gentamicin resistance, generally less than 4%, would suggest that plasmids play little or no role in resistance of *B. cepacia* to the drug.
The exeption to this is C1860 with a curing rate of 66%. In addition the confirmatory evidence of loss of the 185 kb plasmid in C1860 and the 224 kb plasmid in NCPPB 2993 strains cured of gentamicin resistance would suggest that plasmids might play a role in gentamicin resistance in these strains.

Loss of plasmids was also confirmed in C116, J543 and NCPPB 3480 corresponding to loss of resistance to β-lactam drugs described previously. The loss of 224 kb plasmid in NCPPB 2993 is associated both with loss of β-lactam and gentamicin resistance. This suggests the possibility of a multi-resistance plasmid. Cured strains could be tested against a wider range of antibiotics to assess the presence of multi-resistance plasmids. It is notable that a strain of NCPPB 3480 had a decrease in the size of the 200 kb plasmid. This decrease in size could be due to the deletionof a gene involved in antibiotic resistance, possibly the loss of a transposon or an integron 'cassette' encoding the resistance.

Higher levels of curing may be obtained by using agents such as ethidium bromide or ultraviolet light than by serial transfer. The use of such agents could increase curing efficiency giving larger numbers of cured strains for analysis. Curing could also be used to analyse other phenotypic properties of plasmids, most notably degradative properties. Growth or failure to grow on minimal media plates containing the putative degraded chemical could be used to assess cured strains of *B. cepacia*

Chapter 7 Conjugal transfer of plasmid DNA in *B. cepacia*

7.1 Introduction

7.1.1 Investigation of conjugal transfer of plasmid DNA

Investigation of conjugal transfer may be carried out by laboratory mating experiments. Filter mating experiments are commonly used, where donor and recipient cultures are deposited together onto a membrane filter. This allows close contact between cells allowing easier pilus formation and transfer. Broth matings may also be used but are less efficient as cells are free in broth and do not have the close contact afforded by the filter. After the filtering process the filter is placed onto a nutrient medium to allow conjugation to occur.

Following the mating the selection for recipient cells containing the transferred plasmid, transconjugants, is made. This is usually achieved by plating cells out on to a medium containing selective agents for the recipient strain and for a plasmid borne phenotype. An example of this would be a selective media containing rifampicin and mercuric chloride where the recipient strain is resistant to rifampicin and the plasmid in the donor confers resistance to mercury. The rifampicin would prevent growth of the donor and mercuric chloride the growth of the recipient; transconjugants with the properties of both the recipient and plasmid from the donor would grow. By dividing the number of transconjugants arising from a mating by the number of donor or recipient cells, it is possible to arrive at the transfer frequency for a particular plasmid for either or both.

7.1.2 Conjugative transfer in the epilithon

Conjugative plasmids have been investigated in a number of natural environments including soils and aquatic environments such as river epilithon (Fry and Day 1990). Epilithon is a community of micro-organisms on the slime layer found on stone surfaces in the aquatic environment. Stones are thought to be initially colonised by bacteria that secrete polysaccharides that form a slimy layer, which is then colonised by protozoae and algae. This community will grow until the micro-organisms are grazed by invertebrates or slough off. A nutrient rich environment for epilithic bacteria occurs as a result of algae fixing carbon dioxide during photosynthesis and secreting organic materials. In addition carbon sources may enter the epilithon from overlying water, particularly in rivers where the current constantly renews the nutrients available to the epilithon. Such an environment leads to both high viable and total bacterial counts. Movement of stones by strong currents following rainfall leads to the removal of the epilithic layer due to stones colliding, the layer then re-grows on 'clean' stones. These properties have made such an environment an excellent candidate for the study of plasmids and their transfer (Fry and Day 1990).

Plasmids may be isolated from the environment endogenously by the use of a suitable selective medium for the plasmid, though such a method does not demonstrate plasmid transfer, or by exogenous isolation. Exogenous isolation involves introducing a culture of plasmid-free recipient to a community of naturally occurring bacteria. Plasmids may be transferred to the recipient, which may then be isolated by growth on a suitable selective medium. Usually recipients are selected on the basis of a resistance phenotype, such as rifampicin, not commonly found in environmental bacteria. This

method therefore isolates those plasmids capable of conjugative transfer. Epilithic bacteria may be recovered by aseptically scrubbing stones with a toothbrush then used in laboratory matings (Burton *et al* 1982), or recipients may be placed on membrane filters placed face down on stones either in microcosms or *in situ* in rivers without disturbing the epilithon (Bale *et al* 1988a). Plasmids isolated exogenously such as the 251 kb mercury resistance plasmid pQM1 have subsequently been used to study transfer within and between a number of bacterial species, both in the laboratory and in *in situ* experiments (Bale *et al* 1988b, Rochelle *et al* 1989).

In this study it was decided to investigate the transfer of broad host range plasmids into *B. cepacia* isolates from other species, between *B. cepacia* isolates and from *B. cepacia* into other species by laboratory matings. It was also decided to investigate the conjugative transfer of plasmid encoded antibiotic resistance as suggested by the data from curing experiments in Chapter 6, and also to investigate the transfer of epilithic plasmids into a *B. cepacia* recipient by exogenous isolation.

7.2 Materials and methods

Materials

i. Chemicals

Mercuric chloride(Sigma Chemical Co., Poole, Dorset) 100mM stock solution in distilled water.

Rifampicin (Sigma Chemical Co.) at 200 mg ml^{-1} stock solution in methanol.

Gentamicin sulphate (Sigma Chemical Co.) at 100 mg ml^{-1} stock solution in distilled water.

Piperacillin (Sigma Chemical Co.) at 10 mg ml^{-1} stock solution in distilled water.

ii. Strains

E. coli DH10B/pQKH6, pQKH6 = 60 kb plasmid encoding mercury resistance (Hill *et al* 1992)

Ps. aeruginosa PAO 2002/RP1; RP1 = 59 kb plasmid encoding a range of resistances to antibiotics including piperacillin at MIC 500 μg ml^{-1} (Grinstead *et al* 1972, Stanisich and Ortuz 1976, Stanisich *et al* 1989)

E. coli J53R recipient, MIC rifampicin = 500 μg ml^{-1}.

B. cepacia recipient and donor strains, antibiotic or mercury resistance phenotypes as outlined in methods.

Flat stones, approximately 10× 10 ×2cm, removed aseptically from the River Taff in sterile polypropylene stomacher bags for isolation of epilithic bacteria as described in the methods.

iii. Media

DST agar plates (Oxoid CM261, Unipath Ltd., Basingstoke, Hants.) containing selective agents as detailed in the methods.

Nutrient agar plates (Oxoid, Unipath).

10 ml Nutrient broth (Oxoid, Unipath)

Plate Count agar plates(Oxoid, Unipath).

i.v. Solutions

Sterile 0.85% saline.

v. Equipment

Seitz filter units and vacuum pump.

Sterile nylon toothbrushes

v.i. Disposable consumables

47 mm diameter, 0.45μm WCN type membrane filters (Whatman, Maidstone, Kent.)

Methods

i. Investigation of broad host range plasmid transfer by conjugation

Conjugative transfer into *B. cepacia* strains has previously been achieved for the broad host range trimethoprim resistance plasmid R 751 from *Ps. aeruginosa* (Lennon and DeCicco 1991), and with the 60 kb broad host range plasmid pQKH6 from *Ps. putida* (Hill *et al* 1992).On this basis it was decided to investigate the conjugal transfer of two broad host range conjugative plasmids by filter mating into *B. cepacia*, between *B. cepacia* strains and from *B. cepacia* to other species. The plasmids chosen were pQKH6, a 60 kb Inc P-1 mercury resistance plasmid isolated exogenously from the River Taff. (Hill *et al* 1992). Conjugal transfer of pQKH6 has been demonstrated into a range of bacterial species including *E. coli, Alcaligenes eutrophus* and, as described above, *B. cepacia.*

The second plasmid used, RP1 is a 59 kb Inc P plasmid encoding resistance to a range of antibiotics including many β-lactam drugs. RP1 was initially described in *Ps. aeruginosa* and conjugal transfer has been demonstrated with a number of species including *E. coli* (Grinsted *et al* 1972, Stanisich and Ortuz 1976, Stanisich *et al* 1989). Piperacillin resistance was used as the plasmid marker, the host strain containing the plasmid having a MIC of greater than 500 μg ml^{-1}

A mating mix was produced by mixing 5 ml of donor culture and 5 ml of recipient culture, both at 5×10^7 ml^{-1} which was then added to a Seitz filter unit containing a sterile membrane filter. The mating mix was filtered onto the membrane filter which was then removed and placed bacteria side up onto a dry nutrient agar plate, then incubated for 24 h at 35° C. Supplemented DST plates were used to select for transconjugants were made and allowed to dry prior to use (overnight). After incubation the filter was placed into 10 ml of fresh nutrient broth and cells resuspended by vortexing for 5 min. Decimal serial dilutions were prepared in sterile saline and spread onto the transconjugant selective medium. As controls donor and recipient cultures were also spread onto selective media. The plates were incubated at 35 °C for up to 48 h and then checked for the presence of transconjugant colonies. Physical transfer of plasmids was confirmed by the Kado and Liu method described in 5.2. . Each experiment was conducted in triplicate with triplicate dilutions .Donor and recipient counts were conducted at the termination of mating by spread plates of serial dilutions of the donor and recipient cultures.

Transfer frequencies were calculated per donor and per recipient. Frequency was calculated by:

$$\frac{\text{Transconjugants/ml of mating mix}}{\text{Donors/ml of mating mix}} \quad \text{Transfer frequency per donor}$$

$$\frac{\text{Transconjugants/ml of mating mix}}{\text{Recipients/ml of mating mix}} \quad \text{Transfer frequency per recipient}$$

The recipient and donor strainsin each set of matings, and the selective markers used, along with any modifications are given in the following pages:

A *E. coli* DH10B/pQKH6 × *B. cepacia* NCIMB 9092

The 60 kb mercury resistance broad host range plasmid pQKH6 was chosen as donor plasmid; *B. cepacia* NCIMB 9092 was chosen as recipient as it is plasmid free and failed to grow on plates containing mercuric chloride at 27 μg ml^{-1}. However NCIMB 9092 grew poorly on rifampicin thererfore prior to use in mating experiments NCIMB 9092 was trained to grow at 100 μg $ml^{-1}$of rifampicin as a selective marker by growing on plates with increasing concentrations of rifampicin, starting at 25 μg ml^{-1} rising to 200. μg $ml^{-1.}$ Selective plates were produced by the addition of mercuric chloride m, to a final concentration of 27 μg $ml^{-1}$and rifampicin .to a final concentration of 100 μg $ml^{-1}$to molten DST agar. Donor counts were conducted on plates containing mercuric chloride at 27 μg $ml^{-1}$and recipient counts on plates containing rifampicin at

100 μg ml^{-1}.

A. ***B. cepacia*** **NCIMB 9092 × *B. cepacia* C93**

B. cepacia **NCIMB 9092 × *B. cepacia* A548**

Transconjugant NCIMB 9092/pQKH6 was used as donor to transfer to plasmid-free *B. cepacia* C93 and A548 recipients. The mercury resistance encoded by pQKH6 was used as the selective marker for the donor, and gentamicin resistance used as marker for the recipient. The MIC of gentamicin to NCIMB 9092 is 25 μg ml^{-1}compared with 250 μg $ml^{-1}$for C93 and over 500 μg $ml^{-1}$for A548. Selective plates were produced containing 27 μg ml^{-1} mercuric chloride and 100 μg ml^{-1}gentamicin. Donor counts were conducted on plates containing mercuric chloride at 27 μg $ml^{-1}$and recipient counts on plates containing gentamicin at 10 μg ml^{-1}

B. ***E. coli*** **DH10B/pQKH6 × *B. cepacia* C93**

E. coli **DH10B/pQKH6 × *B. cepacia* A548**

Selection of transconjugants was based on the mercury resistanceencoded by the plasmid pQKH6, and gentamicin resistance of the recipient strains. Selective plates were produced containing 27 μg ml^{-1}mercuric chloride and 100 μg ml^{-1}gentamicin. Donor counts were conducted on plates containing mercuric chloride at 27 μg ml^{-1}recipient counts on plates containing gentamicin at 100 μg ml^{-1}.

C. ***Ps. aeruginosa*** **PAO2002/RP1 × *B. cepacia* NCIMB 9092**

Selection for RP1, the donor plasmid, was based upon antibiotic resistance, primarily to piperacillin, encoded by the plasmid. The MIC value for piperacillin against

PAO2002/RP1 is in excess of 500 μg ml^{-1}whilst the MIC against NCIMB 9092 is 5 μg ml^{-1}Selective plates were prepared containing piperacillin at 100 μg ml^{-1} and rifampicin at 100 μg $ml^{-1}$to prevent donor growth. Donor counts were conducted on DST plates containing 100 μg ml^{-1}piperacillin, recipient counts on plates with rifampicin at 100 μg ml^{-1}.

D. ***Ps. aeruginosa*** **PAO2002/RP1 × *B. cepacia* C93**

Ps. aeruginosa **PAO2002/RP1 × *B. cepacia* A548**

Piperacillin resistance was used as the selective marker for the plasmid, gentamicin resistance was used for selection of the recipients. Both C93 and A548 have MIC values for gentamicin in excess of 200 μg ml^{-1}but MIC values of 25 μg ml^{-1}or less for piperacillin, whilst PAO2002/RP1 has an MIC of only 1 μg $ml^{-1}$for gentamicin but over 500 μg $ml^{-1}$for piperacillin. Selective plates were produced in DST agar with piperacillin at 100 μg $ml^{-1}$and gentamicin at 100 μg ml^{-1}. Donor counts were made on DST agar containing 100 μg $ml^{-1}$of piperacillin and recipient counts on plates with 100 μg $ml^{-1}$of gentamicin.

E. ***E. coli*** **DH10B/pQKH6 × *E. coli* J53R**

B. cepacia **NCIMB 9092 × *E. coli* J53R**

B. cepacia **C93 × *E. coli* J53R**

In these matings J53R was chosen as a recipient as it will grow on rifampicin at levels up to 500 μg ml^{-1}. Although NCIMB 9092 and C93 will both grow on rifampicin at 100 μg ml^{-1}, both are inhibited at 200 μg ml^{-1}. On this basis selective

plates with mercuric chloride at 27 μg $ml^{-1}$and rifampicin at μg ml^{-1} were produced. Donor counts were made on plates with 27 μg ml^{-1}mercuric chloride, recipient counts on plates containing 200 μg ml^{-1} rifampicin.

ii. Conjugal transfer of antibiotic resistance in *B. cepacia*

On the basis of the curing data suggesting that plasmid encoded antibiotic resistance may occur in some of the *B. cepacia* strains used in this study, it was decided to investigate whether piperacillin resistance putatively encoded by these plasmids could be transfered by bacterial conjugation. For initial studies it was decided to use strains known to contain plasmids and with high levels of antibiotic resistance, particularly strains found to have lost antibiotic resistance/plasmids in the curing studies, as donor strains. Plasmid-free strains with high levels of gentamicin resistance (as a selective marker) but low levels of piperacillin resistance were chosen as recipient strains.

A. Pilot study

The method basically followed that used for transfer of broad host range plasmids, but for the pilot study matings were carried out singly, without donor or recipient counts, but with the appropriate donor and recipient controls to assess whether any transfer of resistance would occcur.. Three strains that had demonstrated plasmid-loss accompanied by loss of piperacillin resistance in the curing studies (in Chapter 6), C23, C1860 and C116 along with C79, J478 and PW7 were chosen as donors. All these strains had a MIC of 25 μg ml^{-1}or greater to piperacillin, with the exeption of PW7 which has a MIC of 10 μg ml^{-1}. All strains had a MIC of 50 μg ml^{-1}or less for

gentamicin. Although NCIMB 9092 was initially used as a recipient, several donors grew on selective plates with rifampicin at 100 μg ml^{-1}. On the basis of this A548 was chosen as a recipient as it has a low MIC with piperacillin (5 μg ml^{-1}), but a high level of gentamicin resistance as a selective marker. C93 was not considered as a recipient due to a relatively high MIC of 25 μg $ml^{-1}$for piperacillin.

Selective plates were produced by adding piperacillin to DST agar to give a final concentration of 20 μg ml^{-1} (10 μg $ml^{-1}$for PW7) and gentamicin at 100 μg ml^{-1}. Evidence of transfer of piperacillin resistance was taken as growth of transconjugants on the selective plates with no growth of donor or recipient controls.

B.Calculation of transfer frequency

Strains chosen from the pilot study for calculation of transfer frequency were mated in triplicate with triplicate counts following the methods used for broad host range plasmids. Donor counts were done on plates containing 20 μg ml^{-1}piperacillin (10 μg $ml^{-1}$for PW7). Recipient counts were conducted on plates containing 100 μg ml^{-1}gentamicin. Physical transfer of plasmids was investigated by the previously described Kado and Liu method.

iii. Exogenous isolation of plasmids encoding mercury resistance from the environment (river epilithon).

It was decided to investigate *B. cepacia* as a recipient for exogenous isolation and transfer of mercury resistance plasmids from the River Taff, an organically polluted river in South Wales. Detection of plasmid encoded resistance to mercury was chosen

on the basis of a number of other mercury resistance plasmids such as the aforementioned pQKH6 and pQM1 being isolated from this river(Fry and Day 1990, Hill *et al* 1992),. A plasmid-free *B. cepacia* rifampicin resistant mutant was selected as a recipient. A mixture of water and sediment and epilithon from scrubbed stones was used as the donor source

The method used a bacterial mating between *B. cepacia* recipient and epilithic bacteria donors from the River Taff (Fry and Day 1990, Hill *et al* 1992). Epilithic bacteria were obtained by scrubbing 3 large, flat stones from the River Taff with a sterile nylon toothbrush in 100 ml of sterile distilled water in a stomacher bag (Burton *et al* 1982). The stone was then removed and the sample placed in a stomacher for 5 min. 3 sediment samples were collected in an upstream direction in sterile universal containers. 10 ml of sterile distilled water was added to the container and the whole vortexed for 5 min. Equal volumes of rock scrubbings and sediment wash sample were then mixed to give a donor sample. In previous work 10^8 epilithon donors, estimated by acridine orange counts, have been mixed with 10^8 recipient cells, filtered and placed on PCA agar for 24 h at 20 °C to mate (Bale *et al* 1987). Previously viable count estimates of 10^6 bacteria/ml in epilithon produced in this way have been made (Burton *et al* 1982). This represents 5 % of the acridine orange count, so around 5 ml of bacterial suspension would be required to give 10^8 cells per mating. Therefore 5 ml of the epilithon was mixed with 10 ml of the recipient (NCIMB 9092), filtered onto a 0.45 μm filter and placed on PCA for 24 h at 30 °C. As controls 5 ml of epilithon and 10 ml of NCIMB 9092 was filtered and placed on PCA. The epilithon was counted on PCA by spread plating of serial decimal dilutions.

Transconjugant selective plates were produced in DST agar with 27 μg ml^{-1}mercuric chloride to select for mercury resistance plasmid from donors and 100 μg ml^{-1}rifampicin and 10 μg ml^{-1}gentamicin to select for the *B. cepacia* recipient. DST plates containing gentamicin and rifampicin were produced to check growth of controls. After the 24 h incubation time the filters were resuspended in nutrient broth by vortexing for 5 min. The resuspended cells were then diluted serially to 10^{-4}. The mating mix was plated in triplicate, epilithon control and NCIMB 9092 recipient control were plated in duplicate onto : PCA agar plates, DST plates containing gentamicin, piperacillin and mercuric chloride, and DST plates containing gentamicin and piperacillin. All plates were incubated at 30 °C for 24 h.

After incubation, growth of bacteria, their numbers and morphology of colonies were noted. Transconjugant colonies were sub-cultured onto DST agar with gentamicin, rifampicin and mercuric chloride and identity with the recipient checked by the API 20 NE system by the method given in Chapter 2.

7.3 Results

7.3.1 Transfer of broad host range plasmids

Transfer of pQKH6 and RP1 were demonstrated into *B. cepacia* NCIMB 9092. Transfer into *B. cepacia* C93 and between *B. cepacia* strains was demonstrated for pQKH6, as well as transfer from *B. cepacia* into *E. coli* J53R. Transfer of RP1 could not be achieved between *B. cepacia* strains or from *B. cepacia* to *E. coli*. No transfer of pQKH6 or RP1 could be detected with *B. cepacia* A548 as a recipient. Transfer frequencies are shown in Tables 4.1 and 4.2. In all cases no growth or less than 10 colonies at 10^0 dilution were found on recipient or donor control plates, indicating that few or no resistant mutants had not evolved.

Physical transfer of the plasmids was confirmed in each mating by the modified Kado and Liu method. Examples are shown in Plates 7.1 to 7.2. Plate 7.1 confirms the presence of pQKH6 in NCIMB 9092 transconjugants, the plasmid being transferred from *E. coli* DH10B pQKH6 donor. Plate 7.2 shows NCIMB 9092 RP1 transconjugants, RP1 being transferred from *Ps. aeruginosa* PAO2002 RP1 donor, and Plate 7.3 confirms the transfer of pQKH6 into *E. coli* J53R from *B. cepacia* C93 pQKH6 donor.

Table 7.1 Transfer frequencies for broad host range plasmids per donor

Donor	Recipient			
	B. cepacia NCIMB 9092	*B. cepacia* C93	*B. cepacia* A548	*E. coli* J53R
E. coli DH10B/ pQKH6	7.8×10^{-2}	8.2×10^{-3}	ND	–
B. cepacia NCIMB 9092/ pQKH6	–	6.5×10^{-3}	ND	2.0×10^{-2}
B. cepacia C93/ pQKH6	–	–	ND	7.6×10^{-6}
Ps. aeruginosa PAO20002/ RP1	2.2	ND	ND	–
B. cepacia NCIMB 9092/ RP1	–	ND	ND	–

ND = Transfer not detected

– = Not performed

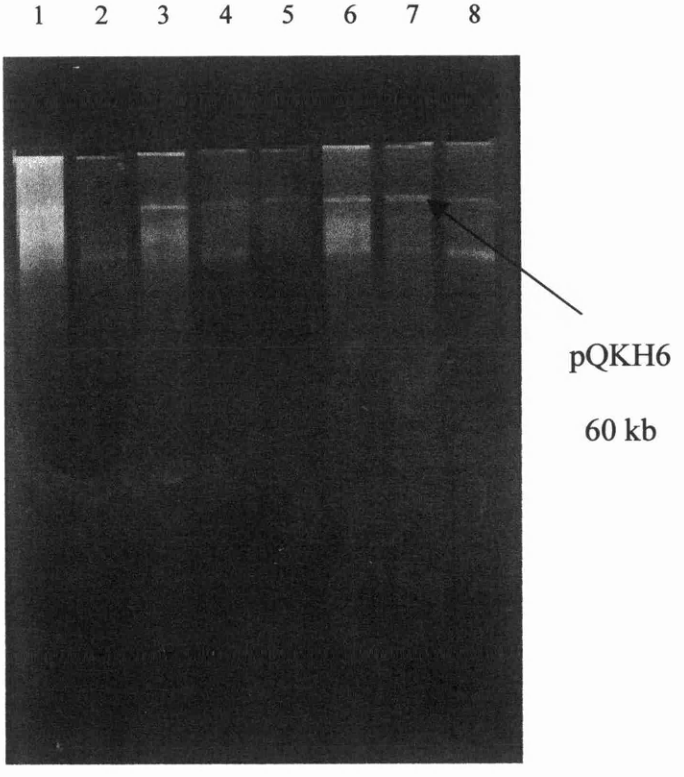
Table 7.2 Transfer frequencies for broad host range plasmids per recipient

Donor	Recipient			
	B. cepacia NCIMB 9092	*B. cepacia* C93	*B. cepacia* A548	*E. coli* J53R
E. coli DH10B/ pQKH6	1.0×10^{1}	2.1×10^{-1}	ND	_
B. cepacia NCIMB 9092/ pQKH6	_	7.3×10^{-3}	ND	2.6×10^{-2}
B. cepacia C93/ pQKH6	_	_	ND	2.0×10^{-5}
Ps. aeruginosa PAO2002/ RP1	3.5×10^{-1}	ND	ND	_
B. cepacia NCIMB 9092/ RP1	_	ND	ND	_

ND = No transfer detected

- = Not performed

Plate 7.1 **Confirmation of conjugal transfer of plasmid pQKH6 from *E. coli* DH10B pQKH6 to *B. cepacia* NCIMB 9092 by modified Kado and Liu method (1981) and agarose gel electrophoresis**



Lane

1 *E. coli* DH10B pQKH6 donor (plasmid obscured through overloading)

2 *B. cepacia* NCIMB 9092 recipient

3 *B. cepacia* NCIMB 9092 pQKH6 transconjugant

4 *B. cepacia* NCIMB 9092 pQKH6 transconjugant

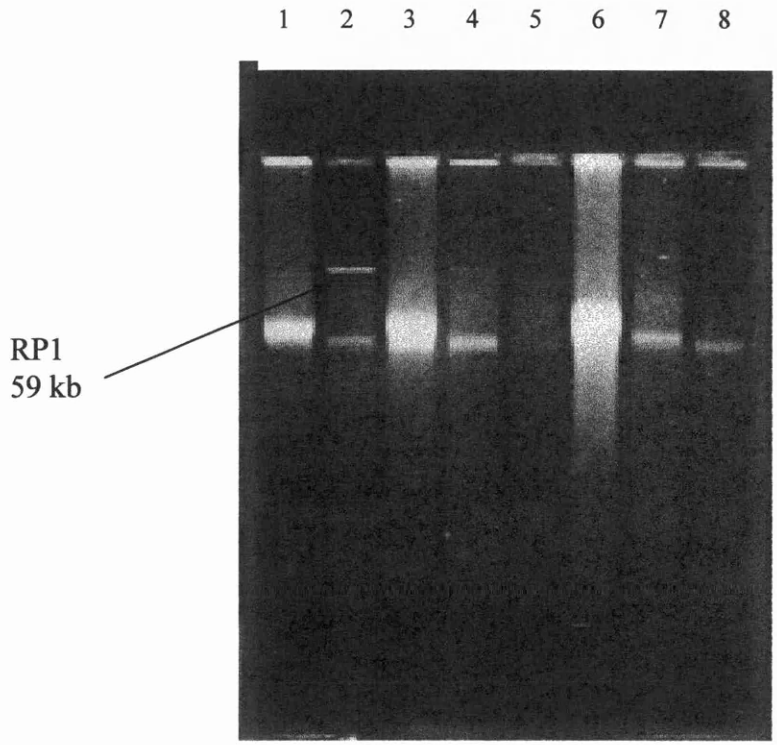
5 *B. cepacia* NCIMB 9092 pQKH6 transconjugant

6 *B. cepacia* NCIMB 9092 pQKH6 transconjugant

7 *B. cepacia* NCIMB 9092 pQKH6 transconjugant

8 *B. cepacia* NCIMB 9092 pQKH6 transconjugant

Plate 7.2 **Confirmation of conjugal transfer of plasmid RP1 from *Ps. aeruginosa* PAO2002 to *B. cepacia* NCIMB 9092 by modified Kado and Liu method (1981) and agarose gel electrophoresis**



Lane

1 *B. cepacia* NCIMB 9092 RP1 transconjugant

2 *B. cepacia* NCIMB 9092 RP1 transconjugant

3 *B. cepacia* NCIMB 9092 RP1 transconjugant

4 *B. cepacia* NCIMB 9092 RP1 transconjugant

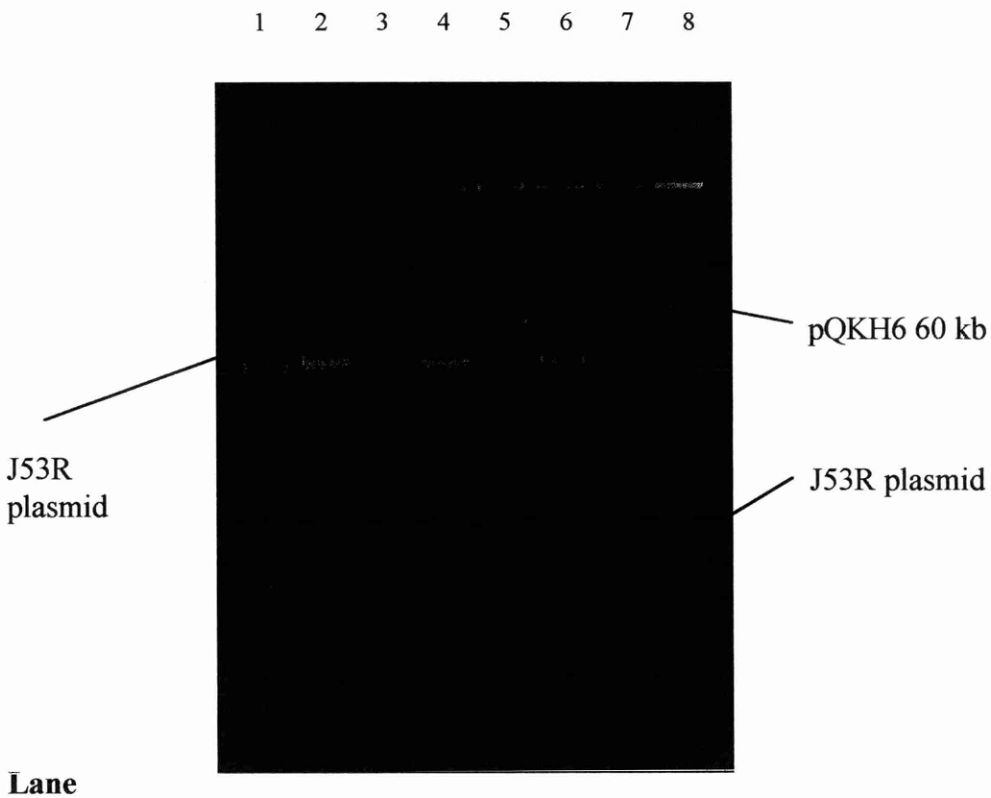
5 *B. cepacia* NCIMB 9092 RP1 transconjugant

6 *B. cepacia* NCIMB 9092 recipient

7 *Ps. aeruginosa* PAO2002 RP1 donor

8 *B. cepacia* NCIMB 9092 RP1 transconjugant

Plate 7.3 **Confirmation of conjugal transfer of plasmid pQKH6 from *B. cepacia* C93 to *E.* coli J53R by modified Kado and Liu method (1981) agarose gel electrophoresis**



Lane

1 *E. coli* 39R plasmid size marker reference strain

2 *E. coli* J53R recipient

3 *B. cepacia* C93 pQKH6 donor

4 *E. coli* J53R pQKH6 transconjugant

5 *E. coli* J53R pQKH6 transconjugant

6 *E. coli* J53R pQKH6 transconjugant

7 *E. coli* J53R pQKH6 transconjugant

8 *E. coli* J53R pQKH6 transconjugant

7.3.2 Transfer of antibiotic resistance between *B. cepacia* strains

The results obtained from the pilot study of conjugal transfer of piperacillin resistance to *B. cepacia* A548 with a range of *B. cepacia* donors indicated thatPW7, C79 and C23 appeared to be the most suitable donors for conjugal transfer of resistance into A548 at an apparently high frequency. On this basis PW7 and C79 were chosen to calculate transfer frequency. The results obtained are shown in Table 7.4.. No colonies were found on either recipient or donor control plates, indicating that resistant mutants had not evolved. In both the pilot study and the triplicate mating used to calculate transfer frequency no physical transfer of plasmid DNA was found either by the Kado and Liu method or Promega Wizard plasmid miniprep for PW7, C79 or C23

Table 7.4 Transfer frequency for conjugal transfer of piperacillin resistance from *B. cepacia* C79/PW7 × *B. cepacia* A548 matings

Donor	Transfer frequency per donor	Transfer frequency per recipient
C79	4.4×10^{-3}	1.7×10^{-9}
PW7	5.7×10^{-2}	2.7×10^{-4}

7.3.3 Exogenous isolation of mercury resistance plasmids from river epilithon with a *B. cepacia* recipient

Epilithon count:

The mean count of epilithon bacteria on PCA was 4.2×10^5 ml^{-1}

Epilithon controls:

PCA - dense growth to 10^{-2} dilution

DST rifampicin/gentamicin - dense growth

DST rifampicin/gentamicin/mercury - growth of around 150 *Pseudomonas*-like colonies at 10^0 dilution.

NCIMB 9092 controls:

PCA - Confluent growth to 10^{-3}

DST rifampicin/gentamicin - Confluent growth at 10^{-4}

DST rifampicin/gentamicin/mercury - Growth on 10^0 , counts of 7, 3 and 12. *B. cepacia* -like appearance

Transconjugant counts

Confluent growth was recorded at all dilutions on both the PCA and DST rifampicin/gentamicin plates.

DST rifampicin/gentamicin/mercury transconjugant selective plates - Growth recorded only at 10^0 dilution. 7, 3 and 12 *Burkholderia*-like colonies were recorded giving a mean count of 7.

API 20NE results:

7 apparent transconjugants were tested. All failed to identify the transconjugant as *B. cepacia*, with the profiles differing from that of the recipient NCIMB 9092.

7.4 Discussion

7.4.1 Conjugal transfer of broad host range plasmids

Transfer of the broad host range plasmids pQKH6 and RP1 into *B. cepacia* was achieved. Transfer of pQKH6 both between *B. cepacia* strains and from *B. cepacia* into other species was achieved. The transfer frequency per recipient of pQKH6 into *B. cepacia* NCTC 10661 from a *Ps. putida* donor has been reported as 1.0×10^{-1} (Hill *et al* 1992). High levels of transfer frequency were obtained in this study with a rate of 1.0×10^{1} per recipient into NCIMB 9092 and 2.1×10^{-1} into C93. A high frequency of transfer was also achieved between NCIMB 9092 pQKH6 transconjugants as donor and *B. cepacia* C93 recipient. The frequencies per donor were 6.5×10^{-3} and 7.3×10^{-3} at per recipient. Transfer of pQKH6 from NCIMB 9092 to *E. coli* J53R also occurred at a fairly high rate 2.6×10^{-2} per recipient, and 2.0×10^{-2} per donor. Transfer could also be detected from C93 pQKH6 to *E. coli* J53R, though the transfer rates were somewhat lower, in the region of 10^{-5} to 10^{-6} both per donor and recipient.

Transfer of the antibiotic resistance plasmid RP1 was also demonstrated at a high frequency into NCIMB 9092, though unlike pQKH6 no transfer into C93 could be detected, either directly from the *Ps. aeruginosa* donor or from NCIMB 9092 transconjugants. It was also notable that A548 was a poor recipient with no transfer of either plasmid detected, though conjugal transfer of antibiotic resistance from other *B. cepacia* strains was apparently achieved. It is notable that this strain demonstrates a very high resistance to aminoglycosides and it is possible that the cell membrane

structure in this particular strain may inhibit transfer from other species or distantly related strains, possibly by inhibiting pilus attachment.

The mating experiments succeeded in their main aim in demonstrating conjugal transfer of plasmid-encoded phenotypic traits into *B. cepacia,* between *B. cepacia* strains and from *B. cepacia* to other species. Transfer of plasmids into *B. cepacia* has previously been reported (Lennon and DeCicco 1991, Hill *et al* 1992), as has transfer between *B. cepacia* strains (Lennon and DeCicco 1991). The transfer rates between *B. cepacia* strains and from a *Ps. aeruginosa* donor into *B. cepacia* strains for the broad host range trimethoprim resistance Inc-P plasmid R751 reported by Lennon and DeCicco (1991) per donor, were lower than those described for Inc-P plasmids in this study. Lennon and DeCicco (1991) found a transfer rate of 10^{-4} to 10^{-7} from *Ps. aeruginosa* compared with rates of 10^{-1} and higher for RP1 in this study, and values of 10^{-5} to 10^{-7} between *B. cepacia* strains compared to around 10^{-3} for plasmids in this study. There are few reports of plasmid transfer from *B. cepacia* to other species. In this study we have demonstrated transfer of the mercury resistance plasmid pQKH6 to an *E. coli* recipient at rates per donor and per recipient in the region of 10^{-2} to 10^{-6}. Conjugal transfer of other mercury resistance plasmids to other species from *B. cepacia* donor strains has been previously demonstrated (Rochelle *et al* 1988, Sabate *et al* 1994). pQM3 had a transfer frequency of 1.9×10^{-2} from *B. cepacia* to a *Ps. putida* recipient, but was somewhat lower (10^{-4} to 10^{-8}) for other *Pseudomonas* recipients (Rochelle *et al* 1988). pAMJ3 transferred into *Ps. putida* recipients at a similar rate (10^{-2} to 10^{-7}), but was lower for a range of other recipients (Sabate *et al* 1994). Other groups have

had little success in the conjugal transfer of plasmids, particularly antibiotic resistance plasmids, to other species (Hirai *et al* 1982).

It is of significance that plasmid transfer by conjugation can occur in both environmental isolates and in clinical isolates such as C93. Although in this study the antibiotic resistance plasmid RP1 could not be transferred to C93, the potential of plasmid transfer, particularly antibiotic resistance, in clinical isolates of *B. cepacia* is of concern. This point was raised by Lennon and DeCicco (1991), who transferred an antibiotic resistance plasmid into and between *B. cepacia* strains. The fact that conjugal transfer can occur in environmental strains adds to the potential of *B. cepacia* as a degradative bacteria, as genes encoding for degradative pathways, or steps in such pathways may be transferred on conjugative plasmids.

Study of conjugative transfer in *B. cepacia* is somewhat difficult. The high levels of antibiotic resistance makes the use of antibiotics as selective markers difficult, the diverse nutritive properties make the use of auxotrophic markers difficult as *B. cepacia* can utilise a wide range of substrates and the difficulties in isolating plasmid DNA (as outlined in Chapter 5) make confirmation of transfer rather tricky. Such problems would appear to be a major factor in the study of plasmids in *B. cepacia.* A number of groups have transformed *B. cepacia* plasmid DNA, usually obtaining the DNA by equilibrium gradient ultracentrifugation, into other species such as *E. coli* for ease of use in studies such as mating, incompatibility and studies into plasmid phenotypic properties (Hirai *et al* 1982, Sabate *et al* 1994, Haak *et al* 1994, Bhat *et al* 1995)

7.4.2 Transfer of piperacillin resistance between *B. cepacia* strains

. Although apparent transfer of resistance was found, this was not accompanied by any evidence of the physical confirmation of plasmid transfer. The transfer frequency from PW7 was reasonably high, 5.7×10^{-2} per donor, and $2.7x\ 10^{-4}$ per recipient. The frequency was somewhat lower for C79, around 10^{-3} per donor and as low as 10^{-9} per recipient,a frequency so low that in effect no transfer has occured. The huge differences between values between frequencies per donor and per recipient could be largely due to the failure to obtain accurate estimations of donor counts in plates containing piperacillin, and in counts of transconjugant colonies where an inoculum effect had apparently occured with growth only found where high numbers of bacteria were inoculated.

There are a number of possible reasons for the apparent transfer of resistance with no physical detection of transfer. A simple explanation would be that the plasmids transferred but could not be detected by the methods used. This is certainly possible considering the difficulties described in Chapter 5. A second explanation is that the apparent transconjugants were actually mutants of the recipient or donor cells though growth at high levels at 10^{-1} indicate that an extremely high mutation rate would be required. Inducible resistance, particularly inducible β-lactamases have been described in *B. cepacia* (Baxter and Lambert 1994), so it is possible that inducible resistance has occurred. However as there was no growth of donor or recipient controls on transconjugant selective plates, it seems unlikely that either mutation or induction of resistance has occurred, as growth on at least one set of these plates should have also

occurred It is also possible that high levels of contaminant growth have occurred, though this seems unlikely as several repeats gave similar results. Another possible explanation is that conjugal transfer of a mobile element such as a transposon may have occurred. Transposons have been strongly implicated in the development of multiple antibiotic resistance in Gram-positive bacteria such as streptococci and staphylococci (Scott 1992). The IS element rich genome of *B. cepacia* could easily incorporate a transposon encoding for antibiotic resistance, or indeed the genomic plasticity described by Lessie *et al* (1996), could lead to the incorporation of a plasmid into the genome. In either case no detection of plasmid DNA would be achieved by the methods used.

7.4.3 Exogenous isolation of mercury resistance plasmids from the environment

The exogenous isolation experiments failed with a *B. cepacia* recipient. The putative transconjugants sub-cultured and identified by API 20NE were not *B. cepacia* recipients, but more likely epilithic bacteria resistant to rifampicin, mercury and gentamicin. The high levels of growth on the epilithic bacteria controls suggest the level or combination of selective markers on the recipient (NCIMB 9092) was insufficient to prevent growth of epilithic bacteria. Obviously the experiment is somewhat limited in scope and scale, with only one *B. cepacia* recipient used. Although NCIMB 9092 was demonstrated to be a good recipient in previous matings, it displays only low levels of antibiotic resistance, so a plasmid-free strain with a higher level of antibiotic resistance may be a more suitable recipient. To achieve exogenous isolation of mercury resistance plasmids from epilithic or other bacteria of

environmental origin may require the testing of a wide range of potential recipients, under a broad range of selective conditions, basically selecting the most suitable conditions and recipients through trial and error.

7.4.4 General considerations and future work

Only a small proportion of the *B. cepacia* strains in this study have been investigated as donor or recipient cells for conjugal transfer of plasmids. Further work could investigate other *B. cepacia* strains as donors or recipients, possibly using different selective markers. An example of this is *B. cepacia* NCTC 10661 (NCIMB 9085), an strain used in this study, has been demonstrated as a recipient for pQKH6 (Hill *et al* 1992). A wider range of transfer between *B. cepacia* strains, and between *B. cepacia* and other species could also be investigated. In this study only transfer into *E. coli* was shown, though other groups have shown transfer into other species, notably *Ps. putida* and *A. eutrophus* (Rochelle *et al* 1988, Sabate *et al* 1994), and it would seem appropriate to investigate the transfer of plasmids from *B. cepacia* to *Pseudomonas* and other β-2 bacteria such as *Alcaligenes,* as well as other *Burkholderia* species such as *B. gladioli*. Overcoming problems with selection of suitable markers may prove difficult. A possible method of overcoming this could be the use of a psychrophilic bacterium as a recipient. Selection of the recipient could then be based on the reported inability of *B. cepacia* to grow at low temperatures (Gilardi 1983), and selection of the plasmid based on its phenotype.

In this study although there was apparent conjugal transfer of piperacillin resistance, no plasmid transfer could be detected. Future work could investigate this, particularly the presence of transposons or incorporation of plasmids into the *B. cepacia* genome. The plasmid transferred could be used as the basis of a probe, or PCR primer to detect plasmid DNA incorporated into the genome.

Other than the transfer of broad host range plasmids in *B. cepacia*, only transfer of antibiotic resistance was investigated. Although the transfer of mercury resistance plasmids has been investigated, there is relatively scant information regarding the conjugal transfer of degradative plasmids in *B. cepacia,* though there is rather more information on the transformation of such plasmid DNA (Haak *et al* 1994, Bhat *et al* 1995). Future work could involve investigation of conjugal transfer of such plasmids.

Chapter 8 Typing of *B. cepacia* strains

8.1 Introduction

A number of methods have been developed and used for typing *B. cepacia*. In this study two molecular methods were examined. Macro-restriction analysis, (restriction digestion of the *B. cepacia* genome then followed by pulsed field gel electrophoresis) and PCR ribotyping. These were chosen both for their typing properties and the fact that they may give a comparison of the genetic structure of the strains.

8.1.1 PCR Ribotyping

The method of PCR ribotyping to study *B. cepacia* was first used by Kostman *et al* (1992) and has subsequently been further developed in the USA (Dasen *et al* 1995) and in Europe (Ryley *et al* 1995, 1996). The technique uses PCR to amplify the 16S-23s intergenic spacer (ITS) region of bacterial rRNA operons (*rrn* operons) using primers based on conserved sequences flanking the ITS region . Differences in the number of operons and heterogeneity in the length of the ITS region allows differences between strains to be characterised.

The polymerase chain reaction (PCR)is an *in vitro* technique to amplify specific DNA sequences from a specific DNA template primer(Reviewed by Taylor 1991) .The method used in this study is based on that of Ryley *et al* (1995), itself a modification of the method of Kostman *et al* (1992). Two sets of primers were used, the first based on a sequence conserved in many Gram negative bacteria. The primers react with a range of Gram negative species including *B. cepacia, E. coli, Ps.*

aeruginosa and *Clostridium difficile* (Ryley *et al* 1995). The second set of primers was specific to *B. cepacia*, designed from a specific sequence in a *B. cepacia* CF strain(Ryley personal communication 1996).

The method can be considered to have three main steps. The first is the extraction of DNA from *B. cepacia*. This was achieved by boiling cells with a suspension of Chelex 100, a chelating ion exchange resin. This was originally developed as a method to extract DNA for PCR from forensic samples (Walsh *et al* 1991) and has subsequently been developed for DNA extraction from bacteria. The second stage is the amplification of *B. cepacia* DNA by PCR. The final stage is the analysis of the products by agarose gel electrophoresis. Products may be digested with *Taq* I restriction enzyme prior to running, or through the use of 'Infinity' agarose extender (Appligene Oncor) that may allow analysis without restriction digestion by increasing the gel's resolution (Ryley personal communication 1996). Using the method with *Taq* I digestion Ryley *et al* (1995) found seven different ribotypes amongst isolates from South Wales CF patients (four paediatric ribotypes and three adult). The method was subsequently used to type 8 different ribotypes amongst CF patients in Denmark (Ryley *et al* 1996). In this study it was decided to initially analyse PCR products on agarose gels with the addition of the agarose enhancer, 'Infinity' agarose extender, and to use *Taq* I digests if there was insufficient differentiation between the strains.

..8.1.2 Typing of *B. cepacia* by macro-restriction analysis

The use of restriction enzyme digests of bacterial whole chromosomal DNA followed by pulsed field gel electrophoresis (PFGE) to type bacterial strains uses the same

principles as restriction fragment length polymorphism (RFLP) analysis, though due to the large size of the DNA rare-cutting restriction endonucleases are used to give a reasonable number of fragments. The method has been named macro-restriction analysis (Grouthes and Tummler 1991, Lessie *et al* 1996) or genome fingerprinting (Rodley *et al* 1995).. The digest pattern is then analysed either qualitatively or quantitatively to type strains. Grouthes *et al* (1988) developed genome fingerprinting for *Ps. aeruginosa* isolated from CF patients. Genomic DNA was digested with *Spe*I, *Dra* I, *Xba* I, *Ssp* I and *Nhe* I then separated by FIGE (Field Inversion Gel Electrophoresis) (Smith and Cantor 1987). Degrees of relatedness of strains could be analysed by the differences in the bands obtained. The method was further developed for 32 *Pseudomonas* and related species (Grouthes and Tummler 1991). PFGE was used following digestion with *Asn* I, *Dra* I, *Spe*I, *Ssp* I or *Xba* I. Qualitative analysis can be made by direct comparison of restriction fragment patterns and is usually sufficient to tpye closelt related strains. Quantitative analysis may be made by using the Dice coefficient to assess similarity, and is usually made when there are large differences between strains. Both quantitative and qualitative analysis has subsequently been applied to *B. cepacia* strains infecting CF patients (Pitt *et al* 1996). The basic method of Grouthes *et al* (1988) was used, and the banding patterns were analysed qualitatively and then quantitatively using the Dice coefficient. A total of 366 isolates from 178 CF patients were analysed both using PFGE and ribotyping. Ribotyping gave more that 50 patterns, with 68 isolates being of a single ribotype, termed ribotype 1. The PFGE macro-restriction analysis showed these ribotype 1 strains to have a very similar banding pattern and genetic structure. There were high levels of heterogeneity amongst the restriction patterns of other strains.

8.2 Materials and methods

Materials

i. Chemicals

Chelex 100 resin (Bio-Rad laboratories, Hemel Hempstead, Herts.)

Ethanol (James Burroughs, Witham, Essex)

Molecular Biology grade agarose (Sigma Chemical Co., Poole, Dorset.)

Pulsed Field grade agarose (Sigma Chemical Co.)

Low Gelling Temperature grade agarose (Sigma Chemical Co.)

'Infinity' Agarose Extender (Appligene Oncor, Durham)

Proteinase K (ICN Biochemicals, Thame, Oxon)

ii. Media

Nutrient agar (Oxoid, Unipath, Basingstoke, Hants.)

iii. Strains

B. cepacia strains grown overnight on NA as described in Chapter 2 (p.97)

iv. Solutions

N-lauroyl sarcosinate buffer (Appendix I)

MacFarland standards (BioMerieux, Marcy l'Etoile, France)

TE buffer (Appendix I)

TBE buffer (Appendix I)

SE buffer (Appendix I)

0.5 M EDTA solution (Fisons, Loughborough, Leics.)

v. Primers, enzymes and markers

Taq DNA polymerase 5,000 units ml^{-1} (Appligene Oncor, Durham)

Tris HCl Taq buffer as supplied with enzyme (Appligene Oncor, Durham)

Deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) at 100 mM in water (Promega UK, Southampton, Hants)

Magnesium Chloride 25 mM (Promega UK, Southampton , Hants.)

Primers (oligonucleotides synthesised by MWG-Biotech UK Ltd., Milton Keynes, Bucks.) supplied in water:

Conserved Primer No.1 (C1) at concentration of 39.3 pmol μl^{-1}

5' - TTG TAC ACA CCG CCG GTC A - 3'

Conserved Primer No.2 (C2) at concentration of 54.1 pmol μl^{-1}

5' - GCT ACC TTA GAT GTT TCA GTT C - 3'

Specific Primer No.1 (S1) at concentration of 36.9 pmol μl^{-1}

5'- AGA AGT GGC TAG TCT AAA CA - 3'

Specific Primer No.2 (S2) at concentration of 30.6 pmol μl^{-1}

5' - CAA ATA AAG AGT GCA AGG CTC - 3'

Genome qualified restriction endonucleases, supplied with buffers and acetylated BSA (Promega UK, Southampton ,Hants,):

Xba I at 12 units ml^{-1}, recognition site- T^CTAGA

*Spe*I at 18 units ml^{-1}, recognition site- A^CTAGT

Not I at 10 units ml^{-1}, recognition site- GC^GGCCGC

Pulsed field size markers (Sigma Chemical Co., Poole, Dorset). Marker sizes given overleaf.

Saccharomyces cerevisae CHEF size marker (BioRad, Hemel Hempstead, Herts.). Marker sizes given overleaf.

Pulsed field gel size marker sizes

a. Sigma 0.1 - 200 kb pulse marker:

Fragment	Size (kb)	Fragment	Size (bp)
1	194.0	7	6.6
2	145.5	8	4.4
3	97.0	9	2.3
4	48.5	10	2.0
5	23.1	11	0.6
6	9.4	12	0.1

b. Sigma 225-2200 kb pulse marker (*Saccharomyces cerevisiae* [Strain No.YPH80] chromosomes)

Chromosome	size (kb)	Chromosome	Size (kb)
XII	2200	X	745
IV	1640	XI	680
VII	1120	V	610
XV	1100	VIII	555
XVI	945	X	450
XIII	915	III	375
II	815	VI	295
XIV	785	I	225

c. Bio-Rad CHEF *Saccharomyces cerevisiae* [Strain No. YNN 295] size marker

Band	Size (kb)	Band	Size (kb)
1	2,200	9	680
2	1,600	10	610
3	1,125	11	565
4	1,020	12	450
5	945	13	365
6	825	14	285
7	785	15	225
8	750		

vi. Disposable consumables

CHEF plug moulds (BioRad, Hemel Hempstead, Herts.)

Disposable L-shaped spreaders (Met-Lab, Hawarden, Clwyd.)

0.5 and 1.5 ml 'eppendorf' microcentrifuge tubes.

vii. Equipment

CHEF DRII pulsed field gel electrophoresis system (BioRad, Hemel Hempstead, Herts.)

Autogene II thermal cycler (Grant Instruments, Barrington, Cambs.)

Horizon 58 gel electrophoresis tanks (Gibco BRL., Paisley, Scotland)

Model 250-EX power supply (Gibco BRL., Paisley, Scotland)

Methods

i. Isolation of DNA for PCR

The method used was based on that of Ryley *et al* (1995) with some modifications. Around 10 -20 colonies were picked off a fresh overnight culture on NA, and suspended in 1 ml of TE buffer in a 1.5 ml 'Eppendorf' tube and then harvested by centrifugation,. The buffer was carefully removed and the pellet was resuspended in the remaining buffer by vortexing mixing. 100 µl of distilled water was added and the tube mixed. 100 µl of Chelex suspension, produced by mixing 1 g of Chelex 100 with 9 ml of distilled water , was added and vortex mixed. This was freshly produced for each set of DNA extractions. Care was taken to mix the Chelex suspension at regular intervals to ensure the resin remained in suspension. Next the samples were heated in a boiling waterbath for 5 min, then allowed to cool for a few min, and spun at 13,000 g for 2 min to pellet out resin and cell debris. This was reduced from the 10 min,

recommended by Ryley *et al*, as it was found to be sufficient to completely remove resin and debris whilst giving a better yield of DNA for PCR. The supernatant was removed into a clean tube and used as the DNA source for PCR ribotyping. The remaining DNA was frozen at –70°C.

ii. PCR amplification of *B. cepacia* 16S-23s intergenic spacer DNA

The method used was based on that of Ryley *et al* (1995). 11 bacterial strains were tested in each PCR run with *B. cepacia* C1 and NCPPB 2993 run as controls in each set. A twelfth tube with water instead of *B. cepacia* DNA was also set up as a control. A reaction master mix sufficient for 12 tubes was produced for each pair of primers. The reaction mixture consisted of 999.8 µl of water, 6 µl of each of the 2 primers in a set, 2.4 µl of each dNTP, 120 µl of ×10 Taq buffer, 6 µl of magnesium chloride and 4.8 µl of the Taq polymerase. 95 µl of the master mix was pipetted into 0.5 ml tubes and 5 µl of each template DNA was added. 5 µl of water was added to the final control tube instead of DNA.

The samples were placed into a Grant Autogene II thermal cycler The conditions were altered slightly from those of Ryley *et al* (1995). The preliminary heating step was reduced from 98 °C to 94 °C for 5 min in order to prevent any inactivation of the enzyme using a ' hot start'. After the initial heating/denaturation stage, the cycler was programmed for 30 cycles of three steps; an annealing step at 55 °C for 30 s, followed by an elongation step at 72 °C for 90 s and then a dissociation step at 92 °C for 60 s. After 30 cycles there were three final steps; a final annealing step at 55 °C for 30 s, followed by a prolonged elongation step for 5 min at 72 °C to ensure fully-double

stranded molecules from all products. Finally there was a cooling step at 17 °C until the samples were removed. 10 µl of sample was then taken for analysis by agarose gel electrophoresis.

iii. Agarose gel electrophoresis of PCR products

A high resolution gel equivalent to a 3% agarose gel was produced by adding 150 mg of agarose and 450 mg of 'Infinity' agarose extender to conical flask with 3 ml of ethanol. This was then mixed to form an even slurry. 30 ml of TBE was gradually added, mixing to maintain the even slurry. The agarose was melted by heating over a Bunsen flame. The gel was allowed to cool briefly before pouring in a Horizon 58 gel electrophoresis tank. The gel was allowed to set for 2 h , giving an approximate gel thickness of 5 mm. TBE was added as running buffer. 10 µl of PCR product was mixed with 2 µl of loading buffer before loading onto the gel.

The gel was run at 4 V cm^{-1} for 1 h until the tracking dye had run two-thirds to three-quarters of the length of the gel. The gel was then stained with ethidium bromide and photographed as described in Chapter 5 (p. 177).

Analysis was performed by comparison of the number and sizes of products, initially to the type strain, NCPPB2993, . Isolates were placed in the same ribotype if the number of , and size of products was identical. Where differences, even in the size of a single product, were found then the isolate was placed into a different ribotype.

iv. Preparation of agarose embedded bacterial DNA

Cells were harvested from fresh overnight cultures on NA plates by pipetting 5 ml of SE buffer onto the surface of the plate and agitating with a disposable spreader. The liquid was drawn off and mixed with additional SE buffer to give a turbidity equivalent to MacFarland standard 3, approximately to 9.0×10^8 cells ml^{-1}. Initially MacFarland standard 5 was used as this was closer to the value of 1×10^{10} c.f.u. ml^{-1} used by Grouthes *et al* (1988). This number of cells gave rise to too high a concentration of DNA resulting in only partial digestion and heavy smearing in the subsequent macrorestriction analysis, therefore the concentration of cells was decreased to the turbidity equivalent to MacFarland standard 3. 500 µl of this suspension was mixed with 500 µl of 2 % molten low gelling temperature agarose, maintained molten at 40 °C. The resulting 1 % agarose/cell suspension was pipetted into the plug mould, approximately 100 µl for each plug, taking care to avoid air bubbles.

The agarose was allowed to set at room temperature for 1 to 2 h. The agarose plugs were then removed using the tool provided and placed into 1.5 ml 'Eppendorf' tubes. Proteinase K was added to the lysing buffer at a final concentration of 500 µl ml^{-1}, and 1 ml of lysing solution added to the agarose plugs. The plugs were incubated overnight at 56 °C. After the overnight incubation the lysing solution was removed and fresh buffer added. The plugs were then incubated overnight at 56 °C for a second time.

After the incubation the plugs were removed and washed four times in TE buffer. Plugs were stored at 4 °C in TE buffer for up to 3 weeks.

v. Macrorestriction digestion of agarose embedded bacterial DNA

The initial method of digestion was based on that of Pitt *et al* (1996). Agarose plugs were equilibrated on ice for 1 h in 200 µl of the appropriate restriction enzyme buffer. The buffer was removed and replaced with fresh buffer containing 0.1 mg ml^{-1} acetylated BSA and the restriction endonuclease at the concentration recommended by the manufacturer. In this case 20 units per plug for *Xba* I and *Not* I, and 5 units per plug for *Spe*I. The plugs were incubated for 4 h at 37 °C, after which the reaction was stopped by the addition of 12 µl of 0.5 M EDTA to each plug. Plugs were then washed 3 times in TE buffer before running by PFGE.

Little digestion of the DNA was found by this method. To increase the amount of digestion 50 units per plug with the above conditions was used as recommended by Bio-Rad Laboratories (1992). This increased digestion giving macro-restriction patterns. Increasing the digestion time to 12 to 16 h as recommended by Sambrook *et al* (1989) increased the definition of the digestion patterns. On the basis of these results all digests were then carried out using 50 units /plug of restriction enzyme at 37°C for 14 h. The other conditions were kept to those of Pitt *et al* (1996).

vi. PFGE (CHEF) of digested agarose embedded bacterial DNA

Optimisation of PFGE conditions

The intitial PFGE conditions used were based on those of Pitt *et al* (1996), using a 1.2% running gel in 0.5 × TBE buffer, electrophoresed at 5 V cm^{-1} for 35 h with initial and final pulse times of 5s and 35s maintained at 8°C. These conditions proved unsuitable as many of the genomic DNA were clustered at the bottom of the gel, or had

passed out of the gel. In an attempt to remedy this shorter running times of 18 and 25h were tried but failed to give sufficient separation of the DNA fragments.

Upon advice from the BioRad technical department, a lower gel strength of 1 %, with pulse times ranging from 5 to 100s were used. Initially these gels were run for 15h at 8°C. This greatly improved resolution, which was further enhanced by increasing the running time to 21 h. These conditions were then adopted for PFGE macrorestriction analysis.

PFGE conditions for macrorestriction analysis

The conditions outlined above were adopted for PFGE. A 1% pulsed field grade agarose gel was prepared by mixing 1 g of agarose with 100 ml of 0.5 ×TBE buffer, and heating over a Bunsen flame to melt the agarose. After allowing to cool sufficiently, the gel was poured into the PFGE gel casting mould and allowed to set for 1 h. The CHEF DR II PFGE system was assembled and the gel tank and recirculating pipes filled with 0.5 × TBE buffer. The buffer was maintained at 8°C by recirculation through an iced water bath. Agarose embedded DNA plugs and PFGE size markers were trimmed and inserted into the wells of the gel using a flamed spatula. The plugs were then sealed in the gel with 1 % low gelling temperature agarose. The gel was electrophoresed at 5 V cm^{-1} for 21h with initial and final pulse times of 5 and 100s. After electrophoresis the gel was stained in 0.5 μg ml^{-1} ethidium bromide solution for 30 mins, destained in water for 30 mins, then photgraphed under UV transillumination as described in Chapter 5 (p. 176).

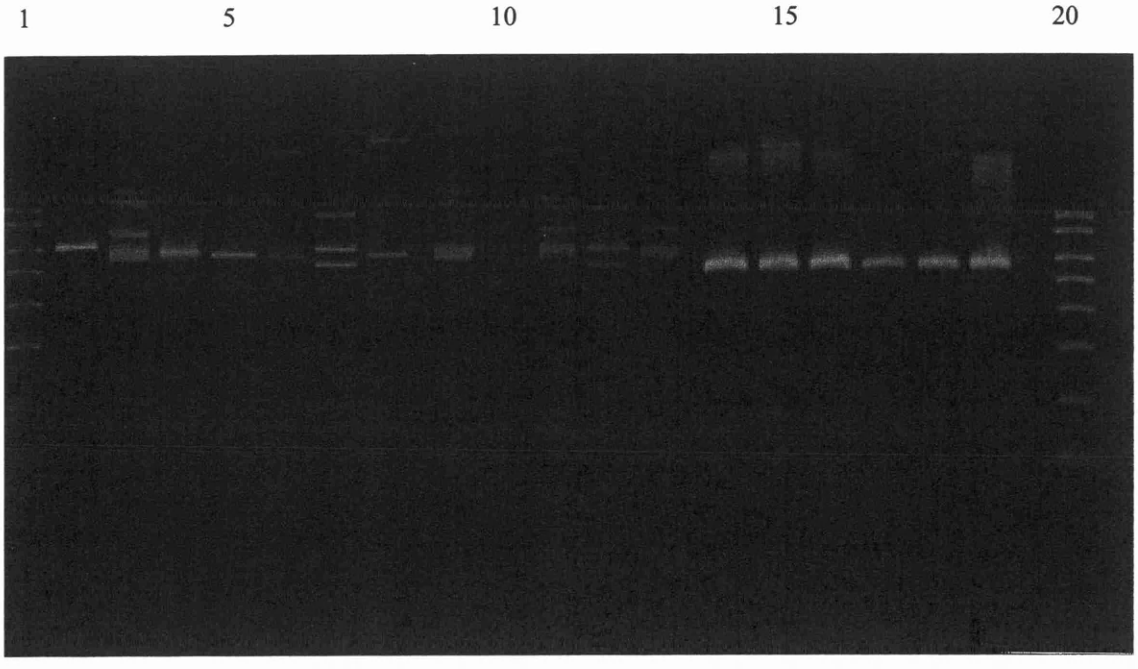
8.3 Results

8.3.1 PCR ribotyping

PCR products were obtained with the conserved primers C1 and C2 though no products were found with the specific primers S1 and S2 Results obtained with the conserved primers were compared qualitatively with each other and with the PCR size marker. Representative results are shown in Plate 8.1. A common pattern was found in all Cardiff CF strains with the exception of C93. These products yielded a strong band, suggesting a high accumulation of product, at approximately 800 bp, with two much fainter bands of around 2,000 and 2,500 bp on agarose gel electrophoresis. The same pattern was also found in the Edinburgh CF isolate C1858, the Strasbourg CF isolate J543 and the Dublin CF isolate D2.

Other clinical strains showed different patterns of PCR products with between 2 to 8 products ranging in approximate size from 700 -2,500 bp. On initial observation several patterns seem very similar, but on closer examination differences in the number of bands or their sizes were found. Every environmental strain gave a different pattern of products. In all 9 different PCR ribotypes were found in the clinical strains and 22 different ribotypes in the environmental strains. 31 different ribotypes were therefore found in a total. The assignment of ribotypes to *B. cepacia* isolates is shown in Table 8.1

Plate 8.1 PCR ribotyping : Composite photograph of representative results



Lanes 1& 20 are PCR size markers

Lanes 2 to 13, except 5, show environmental isolates of *B. cepacia*

Lane 5 & Lanes 14 to 19 show CF isolates of *B. cepacia* of the same ribotype, designated Ribotype cII.

Table 8.1 Assignment of ribotypes to isolates on the basis of PCR ribotyping

Ribotype	Isolates in ribotype
cI	NCPPB2993: Species Type strain
cII	C1,C5, C23, C41, C49, C51, C59, C79, C95, C96, C116, C190, C1858, J543, D2
cIII	C93
cIV	A548
cV	J548
cVI	D3
cVII	D4
cVII	D5
cVIII	D6
cVIX	C1860
cX	NCPPB3480
cXI	NCIMB9088
cXII	NCIMB9092
cXIII	NCIMB9087
cXIV	NCIMB9085
cXV	J2552
cXVI	PW1
cXVII	PW2
cXVIII	PW3
cXIX	PW4
cXX	PW6
cXXI	PW9
cXXII	PW10
cXXIII	PW11
cXXIV	PW12
cXXV	PW13
cXXVI	PW14
cXXVII	PW17
cXXVIII	PW18
cXXIX	PW19
cXXX	PW20
cXXI	A562

8.3.2 Macrorestriction analysis

Following digestion with *Xba* I around 40 fragments of less than 1 kb to over 200 kb were found by PFGE. Initial results were poor, though there was great improvement in later gels. Representative results of *Xba* I macro-restriction are shown in Plate 8.2. The results were in general not sufficiently clear to accurately determine sizes or numbers of bands so quantitative analysis was not possible as the bands were smeared, though qualitative analysis between isolates was possible in many cases.

The results obtained were in close agreement to the PCR ribotypes obatined. Identical or near identical patterns were seen in all Cardiff isolates with the exeption of C93. This macro-restriction pattern was also found in the Edinburgh CF isolate C1858, the Dublin CF isolate D2 and the Strasbourg CF isolate J543 when directly compared to the pattern of Cardiff CF isolates on the same gel. Identity between Cardiff isolates was confirmed by macro-restriction analysis with *Not* I (Plate 8.3).

Different patterns were found with *Xba* I digests of all other clinical and environmental *B. cepacia* isolates with the exeption of the Dublin isolates D4 and D5, which were identical by *Xba* I digestion . The two phytopathogenic strains NCPPB 2993 and NCPPB 3480 were also similar in macro-restriction pattern. Confirmation of the identity between D4 and D5 and differences between NCPPB 2993 and NCPPB 3480 was determined by *Not* I digestion (Plate 8.4). In all 10 digest patterns were found amongst 25 clinical strains tested, compared with 11 PCR ribotypes for the same strains. As with PCR ribotyping no identity was found between the environmental isolates tested.

Plate 8.2 Macrorestriction analysis of *B. cepacia* by *Xba* I digestion followed by PFGE. Composite photograph of representative results.

1 5 10 15 19

Lanes 1 to 9, except lane 2 are CF isolates are CF isolates of Ribotype cII.

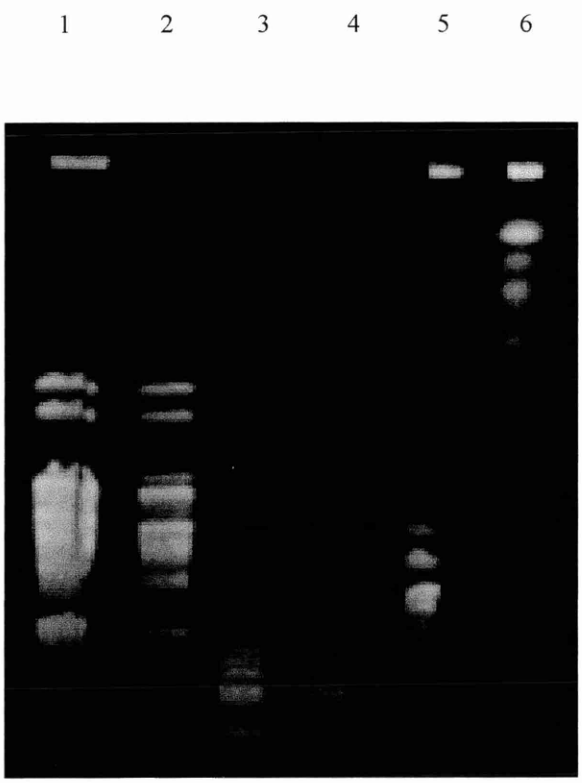
Lane 2 is CF isolate C93 of Ribotype cIII

Lanes 11 & 13 are CF isolates D4 & D5

Lanes 10, 12 & 14 to 17 are environmental isolates

Lane 18 & 19 are size markers

Plate 8.3 Macrorestriction analysis: confirmation of identity by *Xba* I and *Not* I digestion followed by PFGE



lane

1 *Xba* I digested *B. cepacia* C49

2 *Xba* I digested *B. cepacia* C1

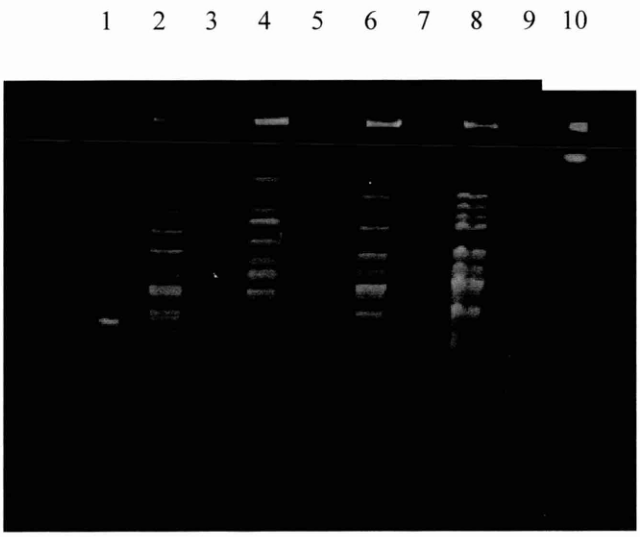
3 *Not* I digested *B. cepacia* C49

4 *Not* I digested *B. cepacia* C1

5 0.1 - 200 kb PFGE marker

6 225 - 2200 kb PFGE marker

Plate 8.4 Macrorestriction analysis: confirmation of identity/non-identity of by *Xba* I and *Not* I digestion followed by PFGE



lane

1 0.1 - 200 kb PFGE marker

2 *Xba* I digested *B. cepacia* NCPPB 2993

3 *Not* I digested *B. cepacia* NCPPB 2993

4 *Xba* I digested *B. cepacia* NCPPB 3480

5 *Not* I digested *B. cepacia* NCPPB 3480

6 *Xba* I digested *B. cepacia* D4

7 *Not* I digested *B. cepacia* D4

8 *Xba* I digested *B. cepacia* D5

9 *Not* I digested *B. cepacia* D5

10 225 - 2200 kb PFGE marker

8.4 Discussion

8.4.1 Typing of *B. cepacia* by PCR ribotyping and macrorestriction analysis

PCR ribotyping with the conserved primers and macro-restriction analysis gave very similar patterns of results. Both methods indicated that 15 of 16 Cardiff CF isolates along with one *B. cepacia* isolate from each of the Strasbourg, Dublin and Edinburgh CF centres are of a common strain type and genetic structure. This was somewhat surprising as the Cardiff CF isolates used in this study had previously been typed to 7 different ribotypes or digest patterns (Ryley *et al* 1995, H. Ryley Personal Communication 1996). These results were achieved by *Taq* I digestion of PCR products, and by using different macro-restriction digest conditions (Pitt *et al* 1996), that used somewhat lower amounts of restriction enzymes with shorter incubation times. It is possible in this study that the conditions used gave a fuller digest pattern. The additional *Taq* I digestion step in PCR ribotyping may detect very slight differences in the *rrn* operon giving typing at a higher level of discrimination not detected by electrophoresis of undigested product.

Typing of *B. cepacia* strains infecting CF patients in Denmark (Ryley *et al* 1996) has been achieved by running undigested PCR products on an agarose/infinity extender gel matrix as used in this study. The technique also detects different ribotypes amongst both other CF isolates and all the environmental isolates tested. The fact that two very different methods give near identical typing of nearly 50 *B. cepacia* isolates, along with results in Chapter 9 showing strains with identical PCR ribotyping and macro-restriction analysis to have identical replicon structures, suggests that the 18 isolates

identical by the typing methods are either identical or very closely related.

PCR ribotyping gave different electrophoresis patterns of products for all the remaining isolates, though all were in the same size range and many had similar patterns. An example of this is the similar patterns seen in the CF isolates A548 and D3 to the environmental isolate J2552 and the phytopathogenic collection strains NCPPB 3480 and NCPPB 2993 which appear to have several PCR products of the same size.

Macro-restriction analysis gave very similar patterns of results to the PCR ribotyping. Most notable was that the same 18 isolates with identical PCR results had identical or near identical macro-restriction profiles and again all the environmental isolates differed. The only significant difference was that 2 Dublin CF isolates , D4 and D5, different by PCR ribotyping showed identity with both *Xba* I and *Not* I digestion. Possibly these strains are very similar in the overall genome but differ at the rRNA genes leading to ribotyping differences.

8.4.2 Significance of typing results

It is perhaps of little surprise that 31 different ribotypes from 48 isolates tested by PCR ribotyping and 30 different macro-restriction patterns from 47 tested isolates were found. Pitt *et al* (1996) found more than 50 different strains from 366 *B. cepacia* CF isolates, with 68 strains of a single type found in various centres. In this study 18 of 25 CF isolates showed a common type, whilst the remainder , with the exeption of 2 Dublin isolates all differed by PCR ribotyping and macrorestriction analysis. The finding of a common type in 4 CF centres suggests that this strain may be of a common

origin. As there is strong evidence of person to person transmission of *B. cepacia* amongst CF patients It is conceivable that these isolates had a common single source and were spread through social contacts such as CF summer camps, meetings or within a single centre at CF clinics. The ET 12 epidemic isolate was found to have been transmitted from a source in Canada to the UK by patients attending summer camps in Canada (Govan *et al* 1993). It is certainly possible this strain was spread in a similar manner.

It is also significant that all the environmental strains both from type collections and isolated in this study were of different strains. This suggests a great diversity of *B. cepacia* in the environment. As described in Chapter 2, only one sample gave more than one strain using different media, though these had different API profiles. Unfortunately the failure of PW21 to grow consistently from frozen stocks prevented confirmation of differences between it and PW17. However the techniques did show the diversity of *B. cepacia* strains in the environment, even amongst samples obtained from within close proximity of each other in South Wales. No identity was found with any clinical strains. High levels of variation amongst environmental populations have also been reported in other studies. Wise *et al* (1996) found 65 unique electropherotypes (ET's) from 217 *B. cepacia* isolates from a 5 km stretch of stream over a period of 32 days in the south-eastern USA. One ET type was found consistently over the survey (64 of 217 isolates). It would be of interest to repeat isolation at a limited number of sites to assess if the same type would be found consistently as in the survey of Wise *et al* (1996). This survey, like this study, shows that there is a high degree of strain variability amongst environmental populations of *B.*

cepacia even within a small area.

8.4.3 Failure of specific PCR primers

No PCR products were obtained through the use of *B. cepacia* specific primers. This was not a problem in achieving ribotyping as the conserved primers have proved more than adequate in this study and others (Ryley *et al* 1995, Ryley *et al* 1996). The most obvious advantage with specific primers is that they positively identify the isolate as *B. cepacia,* as well as typing. Other workers (H. Ryley personal communication 1997) have reported difficulties in the use of these primers with some *B. cepacia* strains. The fact they were designed on the basis of a specific sequence from a CF strain of *B. cepacia* suggests that the sequence may not be conserved throughout the species complex, and so will only react with a limited number of strains, and therefore would be of little use in typing.

Other *B. cepacia* specific PCR primers, including primers to the 16S-23s internal transcribed spacer, have also been found to react poorly with a large proportion of tested isolates (Tyler *et al* 1995). This could be due to variation of the 16S-23s rRNA genes at a species level. The conserved primer sequences that react with a number of Gram negative species may be more suitable for ribotyping.

8.4.4 Problems and improvements to methods

There would appear to be several areas in which the techniques used could be improved and refined, some of which improved as new equipment and techniques to the laboratory became more familiar and better developed . This was certainly the case with macro-restriction analysis. Early gels gave blurred ,poorly separated fragments,

particularly when using the conditions of Pitt *et al* (1996) . Alterations to both digest and PFGE running conditions lead to an improvement, and although the majority of the gels were not sufficiently clear to undertake quantitative analysis described by Grouthes *et al* (1992), later gels such as those in Plate 8.4 were in the order of sufficient clarity to allow quantitative analysis.

The use of *Xba* I or *Spe*I give sufficiently different patterns between isolates with around 40 fragments resulting (*Spe*I has been reported to give 38 fragments with the type strain (Lessie *et al* 1996)). *Xba* I was used primarily as it is less expensive than *Spe*I. *Not* I digests were used to confirm identity. Although more rare cutting enzymes such as *Pac* I or *Swa* I could be used, and the smaller numbers of resulting fragments would make analysis easier, the relatively low cost of *Xba* I makes it more suitable for the analysis of large numbers of samples.

The PCR ribotyping followed by agarose/infinity gel electrophoresis gave sufficiently clear results to type strains. The use of a further digest step, such as the *Taq* I digest used by Ryley *et al* (1995), may allow more detailed typing, though the undigested products were sufficient in this study, particularly in conjunction with macro-restriction analysis, and in studies in Denmark (Ryley *et al* 1996). Although there were initial problems with the PCR, these were largely resolved by decreasing the length of time of the pelleting stage after boiling the cells with Chelex in the DNA purification method. Consistent results were achieved with the Appligene Taq polymerase, though use of an alternative polymerase failed to give any PCR products. It was noticeable that DNA prepared by Chelex extraction began to deteriorate after 2 or 3 thawing and freezings,

This was evident using C1 and NCPPB 2993 DNA as controls. Sharpness of bands and the presence of larger bands were reduced after use on 3 or 4 occasions. Preparation of fresh DNA samples for control strains witheach set of PCR ribotyping overcame this problem.

Both methods typed isolates to the same groups or ribotypes, though the PCR ribotyping was far quicker and easier to perform . However the process of macro-restriction analysis is far less time consuming if agarose embedded DNA is prepared for multiple chromosome analysis(as in Chapter 9) at the same time. Both methods showed great variability in the environmental population of *B. cepacia*, and differences between environmental and CF isolates suggesting that they form a separate population, or possibly species. Identical patterns found amongst CF isolates from different centres strongly support a common source of these isolates and of person-to-person transmission of *B. cepacia* in CF (Govan *et al* 1996)

Chapter 9 Investigation of multiple replicons in *B. cepacia*

9.1 Introduction

9.1.1 Multiple bacterial replicons

PFGE has been used to investigate chromosomes in protozoa and yeasts. Multiple replicons have been described in a number of bacterial species including *Rhizobium* and *Agrobacterium* (Sorbal *et al* 1991, Allardet-Servet *et al* 1993) and *Burkholderia cepacia* (Rodley *et al* 1995, Lessie *et al* 1996).

Two main approaches have been used to investigate multiple bacterial chromosomes. The first of these involves genome mapping by macrorestriction analysis with rare-cutting enzymes. These produce very large fragments of DNA (up to Mb size) that can be mapped into their multiple replicons (Rodley *et al* 1995). Alternatively undigested DNA directly examined by PFGE (Rodley *et al* 1995, Lessie *et al* 1996). Circular DNA molecules in *Leishmania* have been reported to migrate in PFGE in an unusual way (Beverly 1988), and it appears that large circular replicons in bacteria have little or no mobility in PFGE (Lessie *et al* 1996). However multiple replicons may be detected due to small numbers of randomly linearised replicons being formed during the preparation of the agarose embedded DNA plugs. This method was employed by Lessie *et al* to study multiple chromosomes in twelve *B. cepacia* strains using freeze thawing of samples or the use of irradiation to increase linearisation. Rodley *et al* (1995) employed random hit linearisation by irradiation before running undigested DNA on PFGE. Circular replicons remain within the agarose plug

9.1.2 Detection of rRNA genes on multiple replicons

Both Cheng and Lessie (1994) and Rodley *et al* (1995) used Southern blotting hybridisation to detect rRNA genes (*rrn* operons) on multiple replicons in *B. cepacia.* Cheng and Lessie found *rrn* operons on each of the three large replicons in *B. cepacia* ATCC 17616. Rodley *et al* (1995) found one *rrn* operon on the 3.17 Mb and 1.07 Mb replicons and four *rrn* replicons on the larger 3.65 Mb replicon in the type strain. The 222 kb replicon had no rRNA genes suggesting it is a plasmid, and the larger replicons are chromosomal in nature carrying rRNA genes essential to function.

To detect rRNA genes in multiple replicons observed by PFGE, the replicon band was removed and the agarose digested with a hydrolysing enzyme (AgarAce). The product was then used as a DNA source in PCR ribotyping (described in the previous chapter). A similar method was also used to obtain plasmid DNA from the PFGE gel.

9.2 Materials and methods

Materials

i. Chemicals

Pulsed field grade agarose (Sigma Chemical Co., Poole, Dorset)

Molecular biology grade agarose (Sigma Chemical Co., Poole, Dorset)

Chromosomal grade agarose (Bio-Rad Laboratories, Hemel Hempstead, Herts.)

Low gelling temperature agarose (Sigma Chemical Co., Poole, Dorset)

AgarAce agarose digestion enzyme, 0.225 units μl^{-1}(Promega, Southampton, Hants.)

ii. Strains

Agarose embedded DNA of *B. cepacia* and other strains to be tested (as described in Chapter 8, p.250)

CHEF size markers; *Saccharomyces cerevisiae* (Bio-Rad Laboratories, Hemel Hempstead, Herts.) (Sizes given in Chapter 8)

CHEF size markers; *Hansenula wingei* (Bio-Rad Laboratories, Hemel Hempstead, Herts.) (Sizes given overleaf)

Bio Rad *Hansenula wingei* CHEF size markers, sizes of chromosomes:

Chromosome band	Size (Mb)
1	3.13
2	2.70
3	2.35
4	1.81
5	1.66
6	1.37
7	1.05

iii. Solutions

0.5 X TBE

Ethidium bromide for staining (As described in Chapter 5, p.175)

i.v. Equipment

Bio Rad CHEF DRII PFGE system (Bio Rad, Hemel Hempstead, Herts.)

v. Dispoable consumables

CHEF plug moulds (Bio Rad, Hemel Hempstead, Herts.)

Universal containers

1.5 ml microcentrifuge tubes.

Methods

i. PFGE separation of chromosomal DNA

On the basis of the sizes of replicons reported in *B. cepacia,* 0.9 to 3.6 Mb, and following discussion with the Bio-Rad technical helpline and the CHEF applications guide (Bio-Rad 1992) electrophoresis conditions based on the *H. wingei* marker were chosen. A 0.8 % gel was prepared by adding 0.8 g of chromosomal grade agarose to 100 ml of 0.5× TBE in a flask . The gel was melted by heating over a Bunsen flame and allowed to cool for 10 to 15 min before pouring into the gel casting frame. The gel was then allowed to set for 1 to 2 h before trimmed agarose embedded DNA plugs were placed into the wells using a flamed spatula. The plugs were then sealed using molten 0.8 % low gelling temperature agarose and allowed to set and cool, before the gel was placed into the electrophoresis cell of a Bio-Rad CHEF-DRII PFGE system. The cell was filled with 0.5× TBE as running buffer maintained at a temperature of between 5 to 8°C. The gel was electrophoresed at 3 V cm^{-1} for 50h with an initial pulse time of 250 s and a final pulse time of 900 s. After electrophoresis gels were stained with ethidium bromide and photographed as previously described (Chapter 5.,p. 177).

ii. Detection of rRNA genes on *B. cepacia* replicons

PFGE was used to separate multiple replicons. Using a sterile scalpel and under UV transillumination, the replicon bands were cut out and weighed. Slices were also taken from the 'inter band' space, from the edge of the gel as a control and from the agarose DNA plug. The gel slices were cut in half and placed in separate tubes. One half of the

slice was macerated with a sterile pipette tip and by freeze/thawing. This was frozen at -70 °C for storage.

The other half of the slice was digested using the AgarAce enzyme isolated from a marine *Flavobacterium*. The amount of enzyme used was determined by the weight of the gel slice. 0.5 to 1 unit of enzyme was recommended per 100 mg of 1 % agarose) 0.8 units of enzyme was therefore used per half gel slice equating to approximately 200 mg of 0.8 % agarose gel. The gel slice was melted by heating at 70 °C, and then pulse spun in a MSE Microcentaur microcentrifuge. The tubes were transferred to a heating block at 45 °C. 3.5 µl of AgarAce enzyme was added and incubated for 15 min. After incubation the samples were frozen at -70 ° C for future use.

The samples from both methods were then used as the DNA source for PCR ribotyping. The materials and methods used were those described previously in Chapter 8 (p.249) with the modification of adding 10 µl of DNA to 95 µl of reaction master mix, rather than 5 µl.

9.3 Results

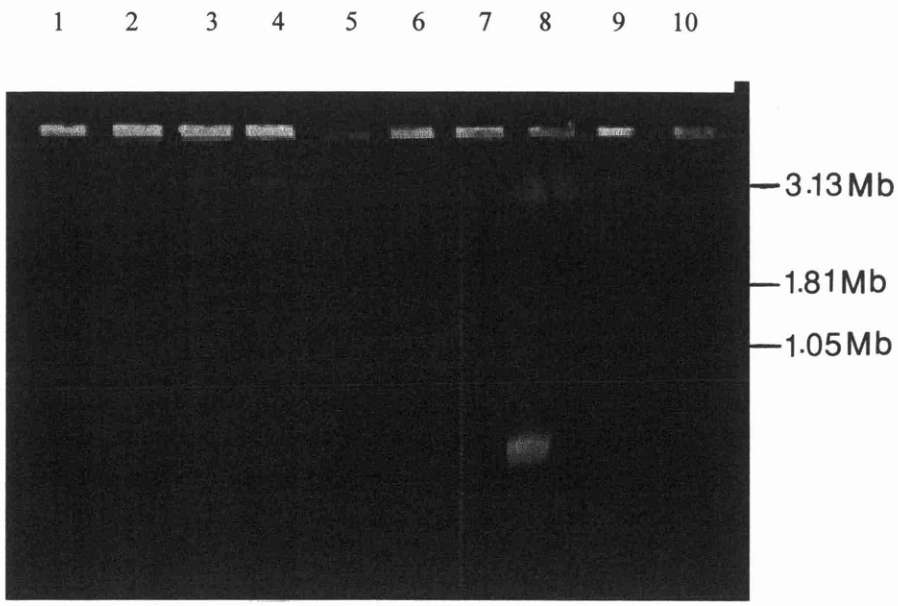
9.3.1 Detection of multiple replicons

Initial attempts to investigate replicon structure in *B. cepacia* were tried using the conditions of Pitt *et al* (1996). A 1.2 % pulsed field grade or molecular biology grade agarose gel electrophoresed at 5 V cm^{-1} for 37 h with initial and final pulse times of 5 s and 35 s with *S. cerevisiae* and/or *H. wingei* markers. Although there was some indication of multiple replicons the methodology was unsuitable leading to poor results, particularly of the larger markers which were insufficiently separated. Several modifications were tried, but after discussion with the Bio Rad technical helpline conditions based on the *H. wingei* marker protocols were tried and were found to give good identification of multiple replicons in the majority of *B. cepacia* isolates tested. The method also indicated multiple replicons in the *B. gladioli* and *Alcaligenes eutrophus* strains tested. Multiple replicons were not detected in *E. coli, Stenotrophomonas maltophilia* or *Ps. aeruginosa.* A selection of representative results are shown in Plates 9.1 to 9.6 .

It is notable that all the Cardiff CF isolates, with exeption of C93, appeared to show a common 2 replicon structure. This structure is also found in strain C190, an isolate sent to clinical laboratories to assess quality control of identification, in the Strasbourg CF isolate J543, the Dublin CF isolate D2 and the Edinburgh isolate C1858. A number of the environmental isolates also show very similar replicon structure. The Dublin CF isolates D4 and D5 also appear to have an identical structure.

A number of strains also show a smaller DNA band. It was thought this may be plasmid DNA or degraded chromosomal DNA. The appearance of a band slightly larger than these in *Ps. aeruginosa* PAO1/ pQM1/R300B appears to correspond with the 251 kb plasmid pQM1, suggesting this band may be large plasmids (100 - 200 kb) in the *B. cepacia* strains, rather than chromosomal fragments.

Plate 9.1 Identification of multiple replicons (chromosomes) *B. cepacia* and other species by PFGE .



lane

1 *B. cepacia* C116

2 *B. cepacia* NCPPB3480

3 *B. cepacia* NCIMB9092

4 *B. cepacia* C79

5 *H. wingei* marker

6 *B. cepacia* PW11

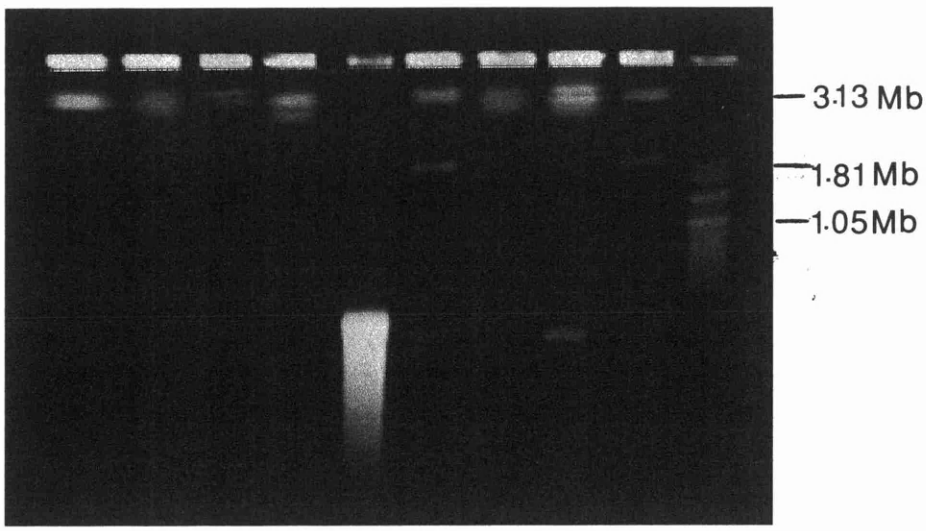
7 *Alcaligenes eutrophus* NCIMB11842

8 *B. cepacia* PW6

9 *B. cepacia* C1

10 *B. cepacia* PW17

Plate 9.2 Identification of multiple replicons (chromosomes) *B. cepacia* and other species by PFGE .



lane

1 *Stenotrophomonas maltophilia*

2 *B. cepacia* NCIMB 9087

3 *B. gladioli* NCPPB 2478

4 *B. cepacia* NCIMB 9092

5 *B. cepacia* J2552

6 *B. cepacia* J543

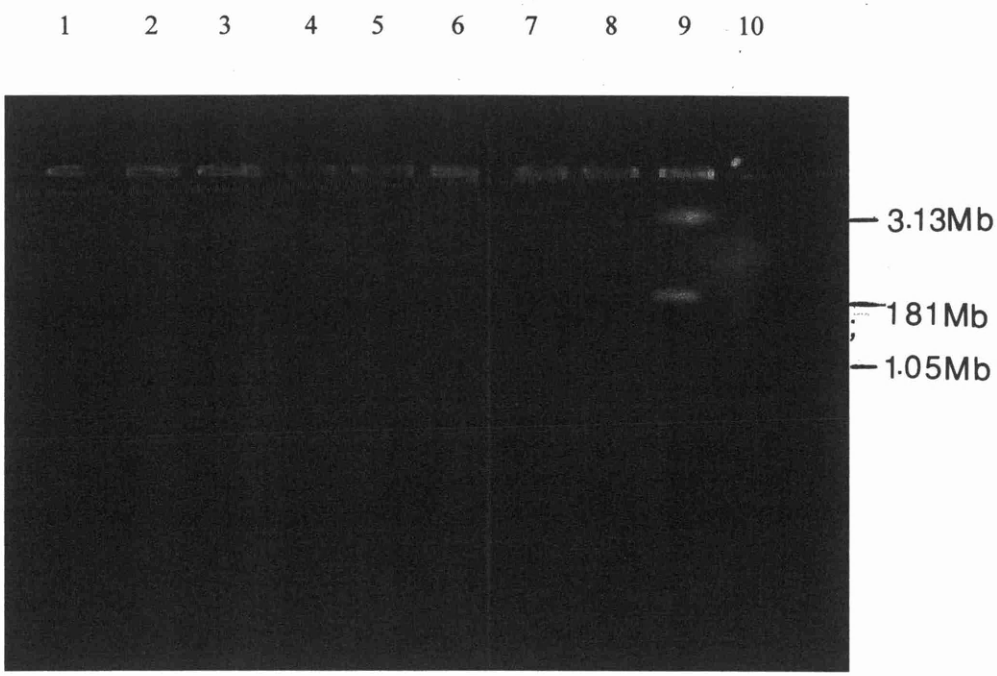
7 *B. cepacia* A562

8 *B. cepacia* D6

9 *B. cepacia* C1

10 *H. wingei* marker

Plate 9.3 Identification of multiple replicons (chromosomes) *B. cepacia* by PFGE .



lane

1 *B. cepacia* C187

2 *B. cepacia* C93

3 *B. cepacia* C23

4 *B. cepacia* C5

5 *B. cepacia* C205

6 *B. cepacia* C190

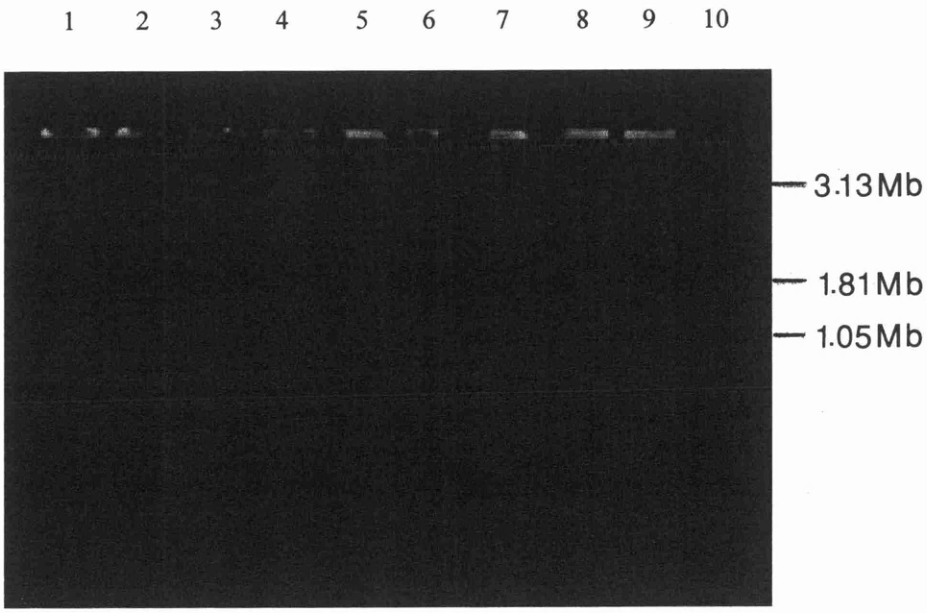
7 *B. cepacia* C11

8 *B. cepacia* C96

9 *B. cepacia* C1

10 *H. wingei* marker

Plate 9.4 Identification of multiple replicons (chromosomes) *B. cepacia* and other species by PFGE .



lane

1 *B. cepacia* PW2

2 *B. cepacia* PW11

3 *B. cepacia* PW7

4 *B. cepacia* PW6

5 *B. cepacia* PW10

6 *B. cepacia* PW17

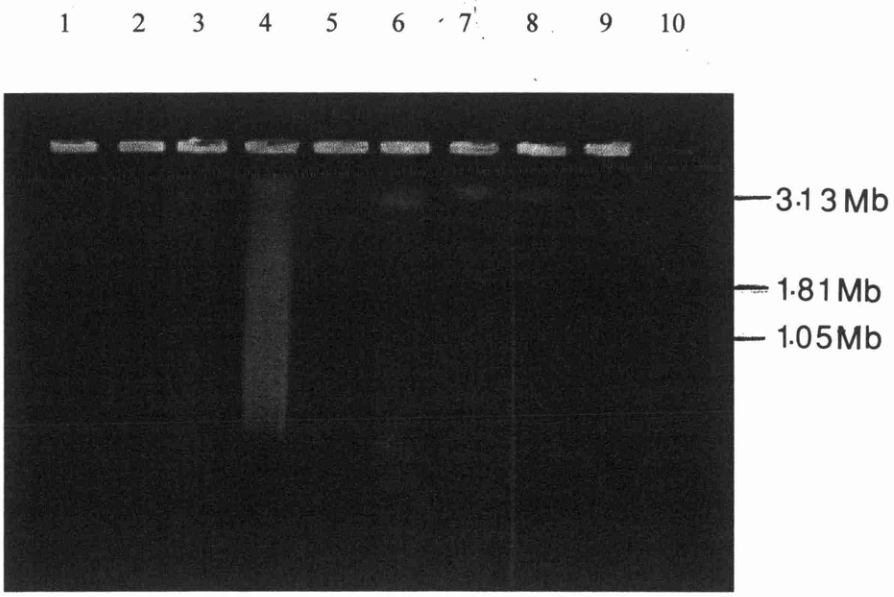
7 *B. cepacia* NCPPB 2993

8 *B. gladioli* NCCPB 2478

9 *B. cepacia* C1

10 *H. wingei* marker

Plate 9.5 Identification of multiple replicons (chromosomes) *B. cepacia* and other species by PFGE .



lane

1 *B. cepacia* PW10

2 *B. cepacia* PW17

3 *B. cepacia* PW14

4 *B. cepacia* PW3

5 *B. cepacia* PW19

6 *B. cepacia* PW6

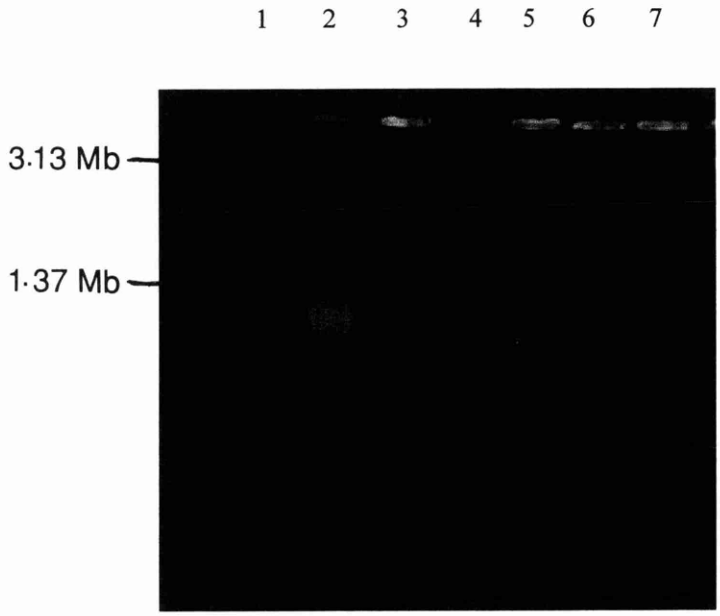
7 *B. cepacia* NCPPB 2993

8 *B. gladioli* NCCPB 2478

9 *B. cepacia* C1

10 *H. wingei* marker

Plate 9.6 Identification of multiple replicons (chromosomes) *B. cepacia* and other species by PFGE .



lane

1 *H. wingei* marker

2 *E. coli* NCTC 10418

3 *Stenotrophomonas maltophilia*

4 *Ps. aeruginosa* PAO1/pQM1/R300B

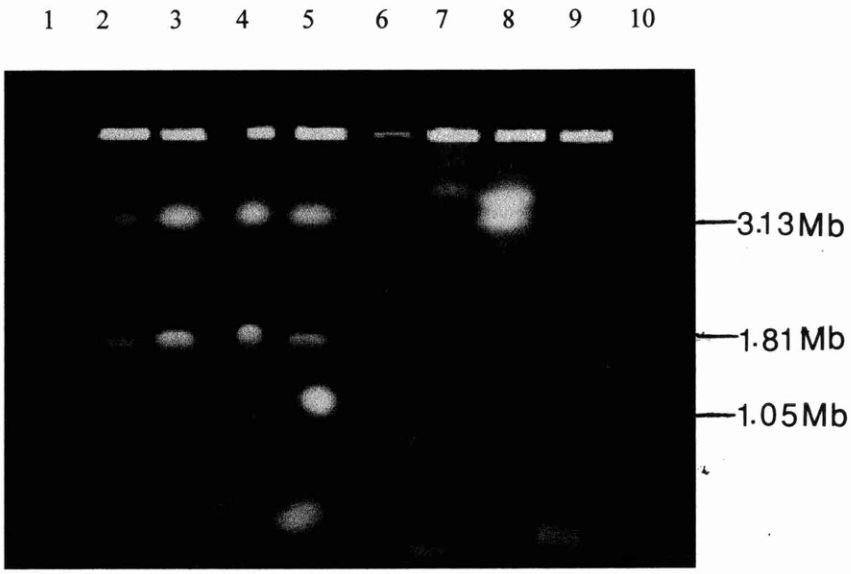
5 *Alcaligenes eutrophus* NCIMB 11842

6 *B. cepacia* C1

7 *B. cepacia* D4

In addition several strains were electrophoreses for an additional 18 h at3 V cm^{-1}, with a pulse time of 900s in an attempt to improve the resolution of replicons in the size range of 2.5 to 3.5 Mb. Plate 9.7 shows a gel electrophoresed for an additional 18 h. The visualisation of the larger replicons such as those in NCPPB 2993 was improved, but to the detriment of visualisation of those in the 1 to 2 Mb region.

Plate 9.7 Identification of multiple replicons (chromosomes) *B. cepacia* by PFGE with an extended electrophoresis time



lane

1 Blank

2 *B. cepacia* C51

3 *B. cepacia* C49

4 *B. cepacia* C116

5 *B. cepacia* C59

6 *H. wingei* marker

7 *B. cepacia* PW4

8 *B. cepacia* NCPPB 2993

9 *B. cepacia* PW8

10 Blank

9.3.2 Size estimation of large replicons in *B. cepacia*

There is no generally accepted methodology for sizing of undigested replicons separated by PFGE. A regression analysis method similar to that used in Chapter 5 for the estimation of plasmid sizes was used to estimate the replicon sizes, using the *Hansenula wingei* CHEF marker as size standard. A number of $\log_{10}$ transformations were tried to obtain a good plot including $\log_{10}$ molecular size against distance travelled in mm, and $\log_{10}$ molecular size against $\log_{10}$ distance travelled. The best results were achieved by plotting molecular size (in Mb) against distance travelled (in mm). The determination of correlation, R^2 value for Plate 9.1 was 99.2 % compared to a R^2 value of 92.7 % for $\log_{10}$ molecular size against $\log_{10}$ distance migrated.

The sizes of the *B. cepacia* replicons were estimated by replacing the y value in the regression equation, the distance migrated of the *H. wingei* standard, with the distance migrated by the *B. cepacia* replicons. The size of the replicon, ×, can then be calculated from the equation.

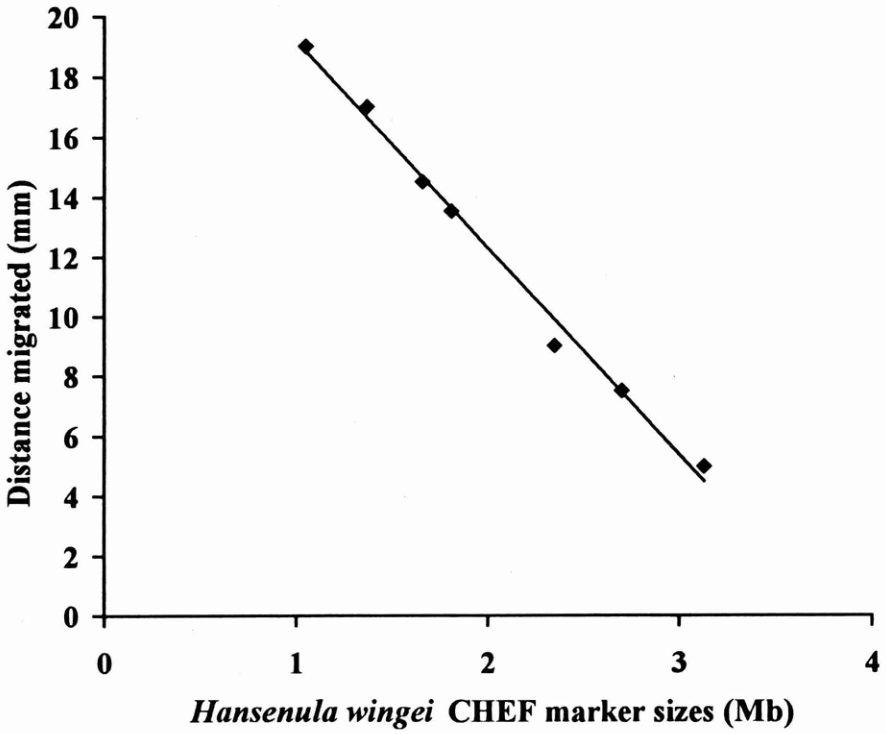
Example calculation

For Plate 9.1, the distances travelled by the *H. wingei* markers (lane 5):

***H. wingei* marker sizes (Mb)**	**Distance migrated (mm)**
3.13	5
2.70	7.5
2.35	9
1.81	13.5
1.66	14.5
1.37	17
1.05	19

The plot of molecular size of marker against distance travelled is shown in Figure 9.1

Figure 9.1 **Plot of molecular size against distance migrated for *H. wingei* marker from Plate 9.1**



From the plot the regression equation was obtained

$y = mc - x$

$y = 26.10 - 6.90\ x$

Where y = distance migrated (mm)

x = molecular size (Mb)

Substituting the values for C1 from Plate 9.1 (lane 9)

For the larger replicon distance migrated = 5 mm

Therefore: $5 = 26.10 - 6.90\ x$

$\frac{5 - 26.10}{-6.90} = x$

$3.06 = x$

Size of replicon = 3.1 Mb

For the smaller replicon distance migrated = 13 mm

Therefore: $13 = 26.10 - 6.90\ x$

$\frac{13 - 26.10}{-6.90} = x$

$1.89 = x$

Size of replicon = 1.9 Mb

As described earlier the line is well predicted with a good degree of fit, the R^2 value being 99.2 %. The above process was repeated for both the other isolates in Plate 9.1, and for replicons in other isolates. The mean R^2 value for the 12 gels used to estimate replicon size was 99.0 %, ranging from 98.2 % to 99.5 % showing good consistency

and correlation for the method, with a consistent and well predicted plot with a good degree of fit.

As with the plasmid size estimation, a number of bands were outside the range of the standards. Again the regression equation was extrapolated to estimate the sizes beyond the standards. This was done on the basis of the good linear relationship between distance travelled, shown by the mean R^2 value of 99.0%, and that the values were only just outside the sizes of the standards.

The method used could effectively estimate the size of the replicons found in this study, with replicons ranging from around 0.5-2.4 Mb. The sizes of replicons in *B. cepacia* and other species determined by regression analysis are shown in Table 9.1. Although in some of the representative gel photographs shown replicons are not seen for some strains, the results given in the table are a mean value that includes several repeats where more clear results for that strain were seen. The frequency distribution of large replicons amongst the *B. cepacia* strains investigated in this study is shown in Figure 9.2.

Table 9.1 Sizes of multiple replicons in *B. cepacia* and other species

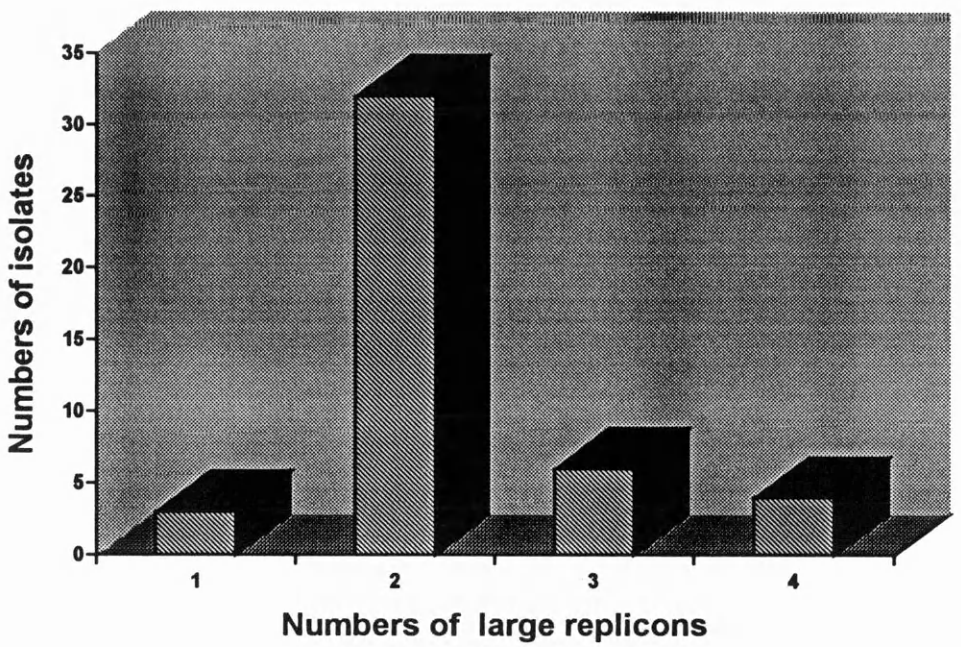
Isolate	Replicon sizes (Mb)	Total genomic size (Mb)
C1	3.1, 1.9 & plasmid (approx.200 kb)	5.0
C5	3.1, 1.9 & plasmid (approx.200 kb)	5.0
C11	3.1, 1.9	5.0
C23	3.1, 1.9 & plasmid (approx.200 kb)	5.0
C49	3.1, 1.9	5.0
C51	3.1,1.9	5.0
C59	3.1, 1.9 & plasmid (approx.200 kb)	5.0
C79	3.1, 1.9	5.0
C93	3.1, 2.3	5.4
C95	3.1, 1.9	5.0
C96	3.1, 1.9	5.0
C116	3.1, 1.9	5.0
C187	3.1, 1.9 & plasmid (approx.200 kb)	5.0
C190	3.1, 1.9 & plasmid (approx.200 kb)	5.0
C205	3.1, 1.9 & plasmid (approx.200 kb)	5.0
J543	3.1, 1.9 & plasmid (approx.200 kb)	5.0
J478	3.2, 3.1, 1.1	7.4
A548	3.1, 2.8, 0.8, 0.7	7.2
A562	3.2, 3.0, 0.8, 0.6	7.6
C1858	3.1, 1.9	5.0
C1860	3.1, 2.5	5.6
J2552	NR	-
D2	3.1, 1.9	5.0
D3	3.1, 2.8, 0.7, 0.5	7.1
D4	3.1, 2.9, 0.7	6.7
D5	3.1, 2.9. 0.7	6.7
D6	3.2, 3.0, 0.6, 0.5	7.3
NCPPB 2993	3.4, 3.1, 1.1, & plasmid (approx.200 kb)	7.6
NCPPB 3480	3.1, 2.6, 0.6 & plasmid (approx.200 kb)	6.3
NCIMB 9087	3.2, 3.0	6.2
NCIMB 9092	3,2, 2.8, 0.8	6.8
PW1	3.0, 2.8	5.8
PW2	3.1	3.1
PW3	3.2	3.2
PW6	3.2, 3.0 & plasmid (approx.200 kb)	6.2
PW7	3.1, 2.9	6.0
PW9	3.2, 2.9	6.1
PW10	3.2, 3.0	6.2
PW11	3.1, 2.9	6.0
PW12	3.2, 2.9	6.1

Table 9.1 (Continued)

Isolate	Replicon sizes (Mb)	Total genomic size (Mb)
PW14	3.1, 1.6	4.7
PW15	3.0, 2.8	5.8
PW17	3.2, 3.1	6.3
PW18	3.2, 2.9	6.1
PW19	3.1, 2.9	6.0
PW20	2.9	2.9
B. gladioli NCPPB 2478	3.2, 3.0 & plasmid (approx.200 kb)	6.2
Alcaligenes eutrophus NCIMB 11842	3.3, 3.0	6.3
Stenotrophomonas maltophilia 4543	3.3	3.3
Ps. aeruginosa PA01/pQM1/R300B	3.1 & plasmid (approx.200 kb)	3.1
E. coli NCTC 10418	NR	-

NR = No result obtained

Figure 9.2 **Frequency distribution of large replicon numbers in *B. cepacia* isolates investigated in this study**



9.3.3 Detection of rRNA genes on multiple replicons by PCR ribotyping

Isolates C1 and D4 were chosen as they gave well defined, well separated chromosomal bands and are of different ribotypes. No PCR products were found with the macerated D4 gel slices. A product was found with the AgarAce digested DNA embedded plug slice but no products were found with any other D4 gel slice. The PCR products were unlike the Chelex-extracted D4 DNA, With C1 there was a small amount with the gel slice with both the macerated and digested samples. There was more PCR product with the digested samples, though as with D4, the PCR products differed from the Chelex-extracted DNA..

9.4 Discussion

9.4.1 Multiple replicons in *B. cepacia*

Between 1 to 4 large replicons were found in the *B. cepacia* isolates tested, with a total genome size in the range of 2.9 to 7.6 Mb. However, when only those isolates possessing multiple replicons were considered then a genome sizes of 4.7 to 7.6 Mb was observed. Lessie *et al* (1996) found a range of total chromosomal DNA size of between 4.6 to 8.1 Mb in 12 *B. cepacia* strains using a similar undigested PFGE technique but only counted replicons of 0.9 Mb and larger in their estimations, considering smaller replicons to be megaplasmids, but in this study all replicons of or in excess of 0.5 Mb were counted in the total genome size, though in both this study and that of Lessie *et al* (1996) these cut-off figures are arbitrary and only affect seven isolates in this study.

43 of the 46 isolates tested (93 %) to possess more than one chromosome. There are three possible reasons for the 3 environmental isolates with single replicons. Firstly the single replicon isolates may have been mis-identified as *B. cepacia,* secondly they may be *B. cepacia* strains with single chromosome and a smaller genome or thirdly they may have replicons identical or nearly identical in size that have not been separated by PFGE. The validity of the final suggestion could be confirmed by two methods. Macrorestriction analysis with more rare cutting enzymes such as *Swa* I or *Pac* I would allow replicon mapping and hence size calculation of the replicon (Rodley *et al* 1995, Lessie *et al* 1996), or an extended PFGE run time may increase the separation of replicons of a similar size.

The replicon sizes obtained in this study for the species type strain NCPPB 2993 (ATCC 25416) are close to those obtained by both Rodley *et al* (1995) and Lessie *et al* (1996) in their studies. In this study chromosomes of 3.4, 3.1 and 1.1 Mb were found. Rodley *et al* found replicons of 3.65, 3.17 and 1.07 Mb by macrorestriction analysis along with a 220 kb plasmid, and sizes of 3.5, 3 and 1 Mb with undigested samples and the 1 Mb replicon could not be readily seen without irradiation of the sample. In this study the corresponding band was faint. Lessie *et al* found 3.5, 3.1 and 1.1 Mb replicons by macrorestriction analysis, with mainly larger sizes of 3.6, 3.4 and 1 Mb found in undigested samples.

The replicon structures in the other isolates tested in this survey have not been previously investigated. The 12 strains described by Lessie *et al* were either environmental or laboratory strains including the 2, 4, 5-T degrading strain AC1100 which contained replicons of 3.7 and 2.6 Mb and the toluene degrading G4 which was found to contain at least 4 chromosomes and at least 4 large plasmids. No multiple chromosomes from strains isolated from CF patients were described and there appears to be no work published as yet regarding such structures in these strains In this study 26 CF isolates were run undigested on PFGE. All were found to contain at least 2 chromosomes. As with the PCR ribotyping and macrorestriction analysis, a distinct pattern was seen in all the Cardiff isolates with the exception of C93. The same 2 chromosome structure (3.1 and 1.9 Mb) was found in the Cardiff CF isolates, the QC isolate C190, the Dublin CF isolate D2, the Strasbourg CF isolate J543 and the Edinburgh CF isolate C1858. This fits the macrorestriction analysis and PCR

ribotyping data, which suggests these isolates, are the same or closely related. C93 also contained a 3.1 Mb chromosome but had a larger second 2.3 Mb chromosome. The 5.0 Mb total genome size was the smallest found in CF *B. cepacia* isolates. The other Dublin, Edinburgh and Strasbourg isolate had sizes of up to 7.6 Mb, with 2, 3 or 4 replicons. All isolates had different sizes or numbers of chromosomes with the exception of the Dublin CF isolates D4 and D5. D4 and D5 were identical by macrorestriction analysis and also showed the same 3.1, 2.9 and 0.7 Mb replicon structure.

Three other type collection strains were also run on undigested PFGE. These were found to contain two or three replicons, but did not have as large a total genome size. Amongst the environmental strains found in this study no more than 2 replicons were found. Generally the genome size in these strains was around 6 Mb with 2 replicons of around 3 Mb, though all strains were shown to differ by PCR ribotyping and macrorestriction analysis. Other than the 3 strains with the single replicon, the other strain that differed was PW14 that had replicons of 3.1 and 1.6 Mb giving a total size of 4.7 Mb.

A smaller replicon was also seen with several strains. The position of the 251 kb plasmid pQM1 in PFGE gels would suggest this is a large plasmid. The band is seen in a number of strains known to contain large plasmids including C1 and NCPPB 2993. However a band is also seen in strains not previously found in this study to contain a plasmid such as C190 and C205. This could be plasmid DNA not detected by the

methods used in Chapter 5 or some fragments of chromosomal DNA, though as it is found consistently in the same strains it appears more likely to be plasmid in nature.

9.4.2 Multiple replicons in other species

A number of bacteria have multiple chromosomes. These include α-proteobacteria such as *Rhizobium* and also β-proteobacteria including a number of *Burkholderia* species. Rodley *et al* (1995) described multiple replicons in the LMG1199 and LMG1201 strains of the β-proteobacterium *Alcaligenes eutrophus*. In this study 2 replicons of 3.3 and 3.0 Mb were found in *A. eutrophus* NCIMB 11842 (synonymous with LMG1199), confirming the observations of Rodley *et al* (1995).

No multiple replicons were detected in *B. gladioli* by Rodley *et al* (1995), despite being the most closely related species to *B. cepacia*. In this study 2 replicons of 3.2 and 3.0 Mb were found in *B. gladioli* p.v *allicola* NCPPB 2478. This suggests that multiple replicons are commonplace in *Burkholderia* species, including *B. gladioli*, and may also be found in closely related β-2 proteobacteria such as *Alcaligenes* and *Ralstonia*, though more distantly related β-proteobacteria such as *Janthinobacterium* and *Herbaspirillium* show the more typical structure of a single circular chromosome.

All the γ-proteobacteria investigated in this study, *E. coli*, *Ps. aeruginosa* and *Stenotrophomonas maltophilia* were found to contain either a single chromosome or, as in the case of *E. coli*, a 'blur' by PFGE.

The presence of multiple chromosomes in both the genus *Burkholderia* and amongst other β-proteobacteria has so far only been studied to a limited extent, with only *B. cepacia* investigated in significant numbers. Future work would require a wider range of *Burkholderia* strains and other β-proteobacteria to be investigated by PFGE to give a better indication of both the frequency of multiple chromosomes and the numbers and sizes of such chromosomes. The same is true of *B. cepacia* strains from CF patients. The majority of clinical isolates in this study were also from a single CF centre, and therefore may represent only a small number of strains that infect CF patients. In the UK alone over 50 different strains infecting CF patients have been found by macrorestriction analysis and by ribotyping (Pitt *et al* 1996), and it is likely that many more strains are found world-wide. A more complete survey of both American and European strains would be needed to be representative of the *B. cepacia* strains pathogenic to man.

9.4.3 Identification of rRNA genes on replicons by PCR ribotyping

The attempt to determine the presence of rRNA genes on replicons by PCR ribotyping was unsuccessful. This could be due to a number of factors. The amount of DNA recovered by maceration or digestion from gel slices may be too small for PCR detection, though the ammounts observed in the gel would suggest there is sufficient DNA in the bands. TheDNA recovered may be too degraded for PCR. The agarose itself, particularly analar grades of agarose, may contain impurities that interfere with the PCR reaction when released by hydrolysis with AgarAce or by maceration of the gel slices (Promega 1996). The use of TBE buffer may also decrease the yield of PCR products by up to two-thirds with AgarAce. The AgarAce, however seems to be more

effective than maceration, as there was some reaction with these samples though this may be a non-specific reaction with degraded DNA from the gel slice.

The detection of rRNA genes (*rrn* operons) on a replicon would confirm it to be of a chromosomal nature. Plasmid DNA does not usually contain *rrn* operons (Summers 1996), so presence of such genes on a replicon would be a better confirmation of a chromosome than size alone to determine a plasmid or chromosome as used by Lessie *et al* (1996), particularly in the 0.5 to 1 Mb size range. Southern blotting of PFGE gels followed by hybridisation with a 16S - 23s RNA gene probe has been used to successfully used to map *rrn* operons on *B. cepacia* replicons (Cheng and Lessie 1994, Rodley *et al* 1995, Lessie *et al* 1996). However such techniques are more difficult and time consuming to perform than PCR and also require the use of radioisotopes and/or other labelling techniques. PCR ribotyping to map *rrn* operons to replicons would be therefore more convenient and quick to perform. A number of modifications to the technique may improve the results sufficiently to allow its use. Freeze thawing or irradiation of samples may increase linearisation of the DNA and hence the amount of DNA running in the gel . Changing the agarose to a preparative grade may reduce any inhibition, particularly if very accurate size estimation is not necessary. An ethanol precipitation step after hydrolysis of the agarose may increase the PCR yield; alternatively TAE buffer could be used (Promega 1996). Increasing the amount of DNA in the PCR reaction may also improve the yield of PCR products.

9.4.4 Identification and sizing of multiple replicons : improvements and alternatives

A number of improvements or alternatives could be made to both the identification and sizing of *B. cepacia* replicons. As described in previous sections increased linearisation either by irradiation or freeze thawing may increase the visibility of chromosomal DNA in PFGE gels. The use of macrorestriction mapping as employed by both Rodley *et al* (1995) and Lessie *et al* (1996) would be beneficial in two ways. Firstly more accurate sizing could be achieved, particularly if there are identical or near identical sized replicons. Secondly it would provide a restriction map of the chromosomes. Although similar techniques were employed in Chapter 8, the enzymes used, primarily *Xba* I, although 'rare cutters' resulted in excess of 30 fragments and are difficult to map or size correctly. Enzymes that cut more infrequently such as *Swa* I and *Pac* I would be more suitable as only a few fragments are produced. For instance ATCC 25416 (NCPPB 2993) has 38 *Spe* I restriction sites, compared with 11 for both *Swa* I and *Pac* I (Lessie *et al* 1996). Their high cost, around £ 100 for 200 units, prevented their use in this study. Only a few *B. cepacia* strains have so far been mapped by this method (Rodley *et al* 1995, Lessie *et al* 1996). Future work, with an appropriate level of funding, could use such methods to map a wider range of *B. cepacia* strains. Used in conjunction with identification of *rrn* operons, this would provide physical maps of *B. cepacia* genomes.

The method used to determine size of replicons was relatively successful with the R^2 value, the correlation coefficient, having a mean value of 99.0 %, indicating a good degree of fit for the molecular size against distance migrated. As with the plasmid size

estimation the biggest problem was estimation of size of the replicons larger than the biggest size standard, although with the PFGE this was mot as significant as the largest replicon was only 0.4 Mb larger than the 3.13 Mb *H. wingei* size marker. As with the plasmid size estimation, extrapolation of the regression equation was used to estimate the largest and smallest replicons. Extrapolation of the regression equation was only used after considering the excellent degree of fit and low variation obtained, and that the values were only marginally larger than those of the standards. This gave sizes of replicons in the type strain (NCPPB 2993/ACTC 24516) consistent with those published (Rodley *et al* 1995, Lessie *et al* 1996). The *H. wingei* marker and electrophoresis conditions used gave good and reliable results over a range of 1 to 3.1 Mb. However there was some difficulty in the 3.1 to 3.6 Mb size range both due to the lack of a direct size comparison described above, and the poor separation of larger replicons in this size range. An increased electrophoretic run time may increase separation of the larger replicons, though this may adversely affect smaller sizes. To overcome the marker problem, a second larger marker such as *Schizosaccharomyces pombe* could be used. This would cover a broader size range as *Schizosacch. pombe* possess chromosomes of 3.5 to 5.7 Mb (Bio-Rad 1992). A set of electrophoretic conditions between those used for *Schizosacch. pombe* and *H. wingei* size markers could be used with both markers on a single gel, or alternatively 2 gels could be run, the first with *H. wingei* marker and conditions suitable for that marker, the second with *Schizosacch. pombe* marker and conditions suited to that. Though this would be more costly and time consuming it should give accurate size determination for all replicon sizes. The *S. cerevisiae* marker could also be used to more accurately determine the smaller replicons.

Despite the problems in accurate size determination the data in this study indicates the vast majority of *B. cepacia* strains have a genome consisting of 2 or more replicons, with 93 % of strains tested in this study having this organisation. The distribution of replicons in *B. cepacia* (Figure 9.1) also indicates that a 2 replicon structure is the most prevalent organisation of chromosomes in *B. cepacia*.

Chapter 10 General discussion and conclusions

10.1 *B. cepacia* in the environment

It would be fair to state that *B. cepacia* is primarily an environmental bacterium, with its natural habitat being soils, waters and vegetation. Recovery rates of around 20% in this and other studies (Butler *et al* 1995) from soil, water and rhizosphere samples are considerably higher than recovery rates from surveys in more 'urban' areas (Fisher *et al* 1993, Mortensen *et al* 1995).

There would appear to be great genetic and phenotypic variability in environmental populations of *B. cepacia*. Variability in phytopathogenicity has been previously described (Gonzalez and Vidaver 1979, Yohalem and Lorbeer 1994, Butler *et al* 1995). Although in this study it was demonstrated that environmental strains can cause *in vitro* maceration of onion tissue, the levels of maceration vary greatly from no apparent maceration, to levels of maceration similar to that of the type strain/pathotype NCPPB 2993.

Genetic variability in an environmental population of *B. cepacia* has been previously reported (Wise *et al* 1996). In this study high levels of genetic variability were found. Each of the 21 isolates obtained from the environment in the course of this study, and the environmental isolates from the various type collections were found to differ both by PCR ribotyping and by macrorestriction analysis. It was notable that no identical isolates were obtained at the same survey site or within the same sample using different isolation media indicating variability of *B. cepacia* even within a sample. Despite

differences by the molecular typing techniques, replicon analysis by undigested PFGE revealed similar replicon structures in the majority of environmental strains with 2 replicons in the region of 3 Mb each. It would appear that this replicon structure is common amongst South Wales environmental strains. The typing differences could be due to rearrangements of the genome or incorporation of genetic material promoted by the IS element rich, highly plastic genome of *B. cepacia*. Future work could investigate the presence of IS elements, either by looking in sequences of *B. cepacia* DNA for the terminal repeats that flank IS elements, the presence of 17 kb (Ferrante and Lessie 1991) and 26 kb (Byrne and Lessie 1994) repeats having been shown in *B. cepacia* IS elements. Alternatively known IS elements could be use as the basis of genetic probes in hybridisation (or PCR) studies to determine their presence. The first method has the advantage of also detecting IS elements from new IS families, or IS elements with sequences differing significantly from known IS element sequences.

Plasmids would appear to be reasonably common in environmental strains of *B. cepacia*, with 11 of 28 strains (39 %) found to harbour plasmids, though this is somewhat lower than the 65 % of the clinical isolates found to harbour plasmids. However greater numbers of strains would need be screened for detailed analysis of the rate at which plasmids are found in environmental and clinical strains. A notable point regarding both the distribution of plasmids and *B. cepacia* in the environment was raised by the results of this study. Considering recovery rates using Mast PC medium alone, the levels of *B. cepacia* found in the polluted industrial regions of S. Wales are considerably higher than those of the unpolluted Brecon Beacons National Park, a 24 % recovery rate compared with 8 %. The plasmid content is considerably higher at

polluted sites, some 71 % of isolates at polluted sites were found to harbour plasmids compared with 13 % amongst isolates from clean sites. This is in agreement with Burton *et al* (1982), who found higher levels of pseudomonads in polluted stretches of the River Ely in South Wales (18 % of total isolates) compared with clean stretches (7 % of isolates), though no significant differences in plasmid content were found between the sites.. It may be that the higher levels of *B. cepacia* found in polluted sites are a result of the highly versatile bacterium occupying an ecological niche due to its ability to degrade a number of chlorinated and organic pollutants that are recalcitrant to breakdown by other bacteria and may inhibit their growth. Plasmids found in isolates at such sites could encode for some of these degradative properties, or for resistance to heavy metals such as mercury that may contaminate soils and rivers in the vicinity of old mineworkings, coking works or other industrial sites. A number of mercury resistance plasmids having been described in *B. cepacia* including one from a strain from the River Taff in S. Wales (Rochelle *et al* 1988). The strains PW2 and PW7 found to contain multiple plasmids were found at pollutes sites; PW2 downstream of the site of abandoned mineworkings and slag heaps, PW7 downstream of the former Coed Ely coking works, a site reported as having high levels of organic and toxic contamination, suggesting the possibility of some of these plasmids encoding for degradation or resistance to toxins. Although, as described in Chapter 5, the levels of plasmid DNA isolated were insufficient to allow restriction mapping (or sequencing), the plasmid bands on the gel electrophoresis were of sufficient quality and clarity to be used Southern blotting and hybridisation either with well characterised degradative plasmids such as NAH or TOL (Day 1982), or with *B. cepacia* degradative plasmids such as TOM (Shields *et al* 1995) or pBAH1 (Haak *et al* 1994). The implication of

the role of IS elements in the degradative ability of *B. cepacia* could also be investigated by future work (Haugland *et al* 1991, Lessie *et al* 1996), particularly in strains such as PW2 and PW7.

10.2 Relationship between clinical and environmental strains of *B. cepacia*

As mentioned in the introduction there is considerable controversy regarding the possibility of the environment acting as a reservoir of *B. cepacia* with potential to cause infection in man. Though some groups have claimed that environmental strains are incapable of causing human infections (Bevivino *et al* 1994), the evidence that *B. cepacia* is the causative agent of 'swamp foot' (Taplin *et al* 1971), and more recent evidence of infections in CF patients from environmental sources (Cazzola *et al* 1996, Amalfitano *et al* 1996) add considerable weight to the need for caution in the use of *B. cepacia* as an agent of biocontrol or bioremediation (Butler *et al* 1995, Govan *et al* 1996).

Phenotypic and genetic variability between clinical and environmental strains of *B. cepacia* has been described by a number of groups (Gonzalez and Vidaver 1979, Yohalem and Lorbeer 1993, Butler *et al* 1995, Vandamme 1995). In this study phytopathogenicity to onion was largely confined to environmental strains , with clinical (CF) strains generally being poor pathogens of onion . Such results were also found by Gonzalez and Vidaver (1979). However a small number of CF strains, most notably 2 strains from the Edinburgh CF centre, demonstrated a high level of pathology against onion. A number of strains from this centre have previously been shown to

cause a high degree of onion maceration *in vitro* (Butler *et al* 1995). Differences in the levels of antibiotic resistance between clinical and environmental strains of *B. cepacia* were also found in this study, with in general lower levels of antibiotic resistance amongst environmental strains. This is in broad agreement with Butler *et al* (1995) who found that MIC levels to a number of antibiotics were higher in clinical strains. It should be noted that considerable differences in the MIC levels to individual antibiotics, or groups of antibiotics were observed for *B. cepacia* CF strains from different centres. Strains from the Edinburgh CF centre had high levels of resistance to aminoglycoside antibiotics, but relatively low MIC levels for β-lactam drugs. The reverse is true in the Cardiff CF centre where in general there were higher MIC levels to β-lactams, but considerably lower MIC values than the Edinburgh isolates to aminoglycosides. This may well be a reflection of prescribing practice at these centres (Govan and Nelson 1992). It is also true to say that virtually all *B. cepacia* strains show intrinsic resistance to many antibiotics, including aminoglycosides. It is certainly possible that the differences observed in antibiotic resistance both amongst clinical populations of *B. cepacia*, and between clinical and environmental strains are as a result of exposure to antibiotics in antimicrobial therapy rather than being due to any fundamental differences between clinical and environmental strains. It would be of interest to investigate whether long-term exposure to antibiotics would lead to the development of acquired resistance in environmental strains, giving equivalent levels of resistance to strains from CF patients.

Genetically the majority (93 % of tested isolates) of both clinical and environmental *B. cepacia* isolates investigated in this study have a genomic structure consisting of 2 to

5 multiple replicons or chromosomes. The use of molecular typing techniques, PCR ribotyping and macrorestriction analysis, reveal considerable variation both within and between clinical and environmental populations of *B. cepacia.* Two main points emerged from these results. Firstly there was no common identity found between any of the clinical and any environmental strains supporting the claims of Bevivino *et al* (1994), though similarity between clinical and environmental strains was found with both the PCR products and macrorestriction digest patterns. This was particularly the case with the Edinburgh CF strains. Secondly, a common molecular type or clone occurs in the majority of Cardiff CF isolates tested in this study, which is also found in isolates from patients attending the Dublin, Strasbourg and Edinburgh CF centres. These results would suggest the isolates have a common source, and have been spread by direct or indirect person to person transmission. There is strong evidence that such transmission occurs amongst CF patients (Millar-Jones *et al* 1992, Govan *et al* 1993, Smith *et al* 1993).

Both clinical and environmental isolates were found to harbour plasmids, though as mentioned previously they would appear to be more common in clinical isolates. Although in this study there is evidence of antibiotic resistance plasmids, the majority of plasmids in *B. cepacia* are described as cryptic, the phenotypic property or properties encoded being unknown. Those that have been described are mostly large degradative plasmids from environmental *B. cepacia* strains. Lennon and DeCicco (1991) described a number of large plasmids (up to 222 kb) in strains of clinical or pharmaceutical origin, and suggested they played a significant role in the antibiotic resistance of such strains, though no phenotypes were assigned. However they failed to

detail that several of the environmental strains harboured plasmids of similar sizes (208 and 212 kb). In this study large plasmids were found in both clinical and environmental strains, many of the largest plasmids (130 kb to in excess of 250 kb) being found in environmental strains though the failure to isolate sufficient plasmid DNA prevented any investigation into whether any plasmids were common to more than one isolate. Future work may develop better isolation techniques so plasmids could be mapped by restriction analysis, or alternatively, a limited number could be isolated (possibly by density gradient ultracentrifugation), and used as probes to investigate relatedness of plasmids in different isolates.

Overall there appear to be a number of genetic and phenotypic differences between and within populations of clinical and environmental strains, adding weight to the suggestions of different genomovars within the species of *B. cepacia*, or even that *B. cepacia* is in fact a number of separate species such as *B. multivorans* (Vandamme 1995, Govan *et al* 1996, Revets *et al* 1996, Vandamme *et al* 1997). The evidence of this study would point to differences between clinical and environmental strains, and that ' environmental *B. cepacia*' is distinct from the strains that infect CF patients. This would suggest that *B. cepacia* in the environment poses little or no risk to CF patients. The evidence in this study suggests that the source of *B. cepacia* infecting CF patients in South Wales is not the local environment but rather via person to person transmission. This is strengthened by the fact that identical or very closely related strains have been found to infect CF patients from a number of centres. However this does not explain where the infective strains of *B. cepacia* originally came from. More detailed PCR ribotyping studies suggest that the Cardiff CF isolates may consist of up

to 7 different ribotypes (Ryley *et al* 1995), the evidence of the 2 typing techniques and replicon analysis used in this study indicates they are extremely closely related, if not identical. Despite the evidence in this study it would be rather naive, and arguably foolish, to firmly conclude that the environment is of no risk to CF patients in terms of acquiring a *B. cepacia* infection, particularly considering recent evidence of *B. cepacia* acquisition from the environment (Govan *et al* 1996, Cazzola *et al* 1996). Extensive phylogenetic surveys of *B. cepacia* throughout Europe and North America would be required to make such a conclusion and it is of interest and possibly concern, that some CF isolates more resemble environmental isolates than those from the Cardiff CF centre. Some of the Edinburgh CF isolates are arguably more closely related to the type strain NCPPB 2993/ACTC 25416 on the basis of PCR ribotyping, macrorestriction digest pattern, antibiotic resistance patterns and phytopathogenicity. In addition, Johnson *et al* (1994) demonstrated a high degree of genetic relatedness between the ET 12 epidemic strain and the type strain by multilocus enzyme electrophoresis. The fact that spontaneous cases of *B. cepacia* infection arise amongst CF patients despite strict segregation of infected individuals would suggest there is an environmental source of *B. cepacia* (Govan *et al* 1996, Cazzola *et al* 1996). Such a source may represent a small sub-population of *B. cepacia* in the environment and that environmental surveys such as those conducted in this study have failed to detect such a group as yet. Though the evidence in this study does not indicate the environment is a source of *B. cepacia* it would appear to be wise advice to proceed with caution in the use of *B. cepacia* as an agent of biocontrol or bioremediation, at least until it is conclusively shown that such *B. cepacia* strains are of no risk to CF patients. An increased phylogenetic understanding of the species, whether as separate genomovars

or indeed species may help in coming to a firm conclusion. The development of animal models, such as cystic fibrosis mice (Wilson and Collins 1992), may allow the pathogenicity of *B. cepacia* strains to be studied and Koch's postulates to be tested and confirm whether environmental strains of *B. cepacia* are pathogenic , something that obviously cannot be undertaken with cystic fibrosis patients. The *cftrm* 1 HGU mutant CF mouse appears to be a possible candidate for such studies as it fails to clear *B. cepacia* or *S. aureus* from the lungs and displays lung disease and mucus retention in response to repeated microbiological exposure (Davidson *et al* 1995).

10.3 Genetic organisation of *B. cepacia*

In this study three main areas of genetic interest were investigated; the presence and biology of plasmids in *B. cepacia*, the organisation and size of the *B. cepacia* genome and the use of molecular typing to determine the relationship of different strains.

From this study and others it is clear that plasmids are commonly harboured by *B. cepacia* strains of both clinical and environmental origins (Gonzalez and Vidaver 1979, McKevitt and Woods 1984, Lennon and DeCicco 1991). Despite difficulties in the isolation of plasmid DNA it would appear that more than half of all *B. cepacia* isolates contain plasmids. 52% of isolates in this study were found to contain plasmids, whilst both Gonzalez and Vidaver and Lennon and DeCicco found plasmids in over 80% of isolates. It is notable that the majority of *B. cepacia* plasmids are large, mostly in excess of 100 kb, but that very few have been assigned a phenotypic trait. This is particularly true of antibiotic resistance plasmids where there are few reports of such plasmids being found in *B. cepacia.* In this study there is strong evidence of plasmid

encoded resistance to β-lactam antibiotics. This was demonstrated by curing studies in Chapter 6 where loss of resistance to the ureidopenicillin piperacillin was accompanied by plasmid loss. Mating experiments also indicated the conjugal transfer of resistance to piperacillin, though transfer of a plasmid could not be confirmed. Transformation of sensitive host bacteria with plasmid DNA accompanied by an increased resistance to piperacillin, or another antibiotic, would confirm an antibiotic resistance phenotype. The failure to obtain sufficient plasmid DNA prevented the use of transformation in this study.

Lennon and DeCicco (1991) suggested the presence of antibiotic resistance plasmids in *B. cepacia*, but did not assign a phenotype to any plasmids. It would appear that the apparent absence of antibiotic plasmids in *B. cepacia* is due to a lack of investigation into such plasmids rather than the plasmids not occurring in the bacterium (Wilkinson and Pitt 1995b). The evidence of plasmids commonly encoding for β-lactamases in *Ps. aeruginosa* (Prince 1986), and the fact that these plasmids are largely of the Inc-P incompatibility group adds weight to the argument that such plasmids may occur in *B. cepacia*. Many plasmids described in *B. cepacia* are Inc-P plasmids (Haak *et al* 1995, Bhat *et al* 1995), and in this study the transfer of Inc-P plasmids in *B. cepacia* has been demonstrated, indeed transfer of the antibiotic resistance plasmid RP1 from a *Ps. aeruginosa* donor to a *B. cepacia* recipient was shown.

Conjugative transfer of plasmids into and between *B. cepacia* strains, and from *B. cepacia* to other species was clearly demonstrated in this study. Though the transfer of broad host range plasmids in *B. cepacia* described above has previously been

demonstrated (Rochelle *et al* 1988, Lennon and DeCicco 1991, Sabate *et al* 1984), the transfer frequencies found in this study were higher ; as high as 10^{-1} per donor for the transfer of pQKH6 from *B. cepacia* NCIMB 9092 to an *E. coli* recipient. In this study transfer into and from *B. cepacia* of both clinical origin, C93, and environmental origin, NCIMB 9092, was demonstrated showing the possibility of horizontal genetic transfer by conjugation both in the environment and in clinical situations. Considering the highly plastic nature of the *B. cepacia* genome, such transferred DNA could be incorporated into the genome; this possibly being the reason why there was no detection of plasmid DNA accompanying the transfer of piperacillin resistance.

One of the most striking features of *B. cepacia* is the size, organisation and variability of its genome. In this study genome sizes of up to 7.6 Mb were found by PFGE, and in other studies genomic sizes of over 8 Mb have been reported (Lessie *et al* 1996). This is considerably larger than other Gram negative bacteria, for example the genome of *Helicobacter pylori* (Tomb *et al* 1997) is around 1.7 Mb and that of *E. coli* K-12 is around 4.6 Mb (Blattner *et al* 1997). The complexity of the genome is also unusual, organised into between 2 to 4 circular chromosomes. As outlined in both the introduction and Chapter 9, this occurs in a number of species, including the α-proteobacteria *Agrobacterium* and *Rhizobium.* Rodley *et al* (1995) found multiple chromosomes by PFGE in *B. cepacia* , *Alcaligenes eutrophus* and other *Burkholderia* species, though not in *B. gladioli.*

In this study 45 of 48 *B. cepacia* isolates tested were found to contain 2 or more chromosomes by PFGE. This, along with the description of multiple chromosomes in

12 environmental *B. cepacia* strains shown by Lessie *et al* (1996), would indicate that this is feature of both clinical and environmental *B. cepacia*. The evidence of Rodley *et al* (1995), along with the discovery in this study of chromosomes of 3.2 and 3.0 Mb in *B. gladioli* NCPPB 2478 and the confirmation of 3.3 and 3.0 Mb chromosomes in *A. eutrophus* NCIMB 11842 would suggest that multiple chromosomes are a feature of the genus *Burkholderia*, and may be common throughout β-2 proteobacteria.. The failure to detect multiple chromosomes in *E. coli, Ps. aeruginosa* or *Stenotrophomonas maltophilia* would indicate that this is probably not a feature of γ-proteobacteria.

Considerable variation in the size of the genome and numbers of chromosomes was found both by Lessie *et al* (1996) and in this study. The size of the genome would appear to vary between around 4 to 8 Mb, with 2 to 4 replicons of less than 1 Mb to more than 5 Mb in size. This large genome size may be a consequence of the high plasticity of the IS element rich genome of *B. cepacia*. The acquisition of 'foreign' DNA such as plasmids or transposons mediated by the IS elements may lead to an increasing genome size, This plasticity may also play a major role in the wide ranging nutritive and degradative properties of *B. cepacia*. There is strong evidence that genes involved in the degradation of 2, 4, 5-T were acquired in such a manner (Lessie *et al* 1996). However this does not explain the differences in chromosome numbers. Although there is evidence that smaller replicons may recombine to form larger structures (Lessie *et al* 1996), no common macrorestriction fragments were found in the 3 replicons of the type strain ACTC 25416 (NCPPB 2993) during physical mapping with very rare cutting restriction enzymes. This lack of a common genetic sequence would indicate that the chromosomes are not deleterious derivatives of a

larger chromosome ,or fragments of a larger chromosome broken in sample processing (Rodley *et al* 1995, Lessie *et al* 1996). This would suggest that the *B. cepacia* genome consists of a number of distinct chromosomes, with genes on each chromosome encoding essential functions. The assignment of essential genes to different replicons, particularly the assignment of rRNA genes to each of the 3 major replicons in the type strain supports this statement (Cheng and Lessie 1994, Rodley *et al* 1995, Lessie *et al* 1996). It has been suggested that the chromosomes may behave rather like a number of large plasmids, rather than like the more usual 'single' chromosome. Each chromosome may have a different replication control mechanism, and it may be possible to transfer whole chromosomes between cells by conjugal transfer in the manner of a plasmid (Lessie *et al* 1996). Such a situation requires a firm definition of a plasmid, particularly in view of smaller chromosomes of less than 1 Mb. It may be argued that a plasmid should be defined as a replicon carrying genes non–essential to growth in 'normal' or 'good'conditions but gives the advantage of allowing growth in certain circumstances such as the presence of antibiotics or heavy metals, rather than on the basis of size employed by Lessie *et al* (1996). As mentioned in Chapter 9, the mapping of rRNA genes to a replicon, either by hybridisation or , as attempted in this study, by the use of PCR with primers for the rRNA genes, would allow confirmation as a replicon of a chromosome rather than a megaplasmid.

The reason for the multiple chromosome structure is unknown, though the rather neat theory that the large genome of *B. cepacia* makes it advantageous to have such a structure has been put forward (Lessie *et al* 1996). The time required for the replication of several small circular replicons, particularly if each has an independent

control mechanism, would be less than for a single large replicon; important for a (relatively) rapidly proliferating bacterium such as *B. cepacia*. Smaller replicons may allow greater fidelity of replication, and if under independent control, greater flexibility in the control of replication and segregation of chromosomes in cell division. More detailed information regarding the organisation of genes upon the replicons, their replication and replication control, and how chromosomes segregate in cell proliferation would be required to understand the biology and the requirement for such genetic organisation in bacteria.

10.4 Conclusions

A number of conclusions may be reached on the basis of the results in this study. Firstly that *B. cepacia* has a large genome of up to 8 Mb that is highly variable and arranged into between 2 to 4 chromosomes of varying size. This arrangement was observed for both clinical and environmental strains. Multiple chromosomes were found in both *B. gladioli* and *A. eutrophus* in this study, and along with the results of Rodley *et al* (1995), this would indicate that such genetic arrangements are common in the genus *Burkholderia* and possibly throughout β-2 proteobacteria.

Plasmids are commonly found in *B. cepacia*, particularly large plasmids, but appear to be more common in clinical strains. It also appears that plasmids are more commonly found in environmental strains from polluted sites than in 'clean' sites. There is strong , but not conclusive, evidence of antibiotic resistance plasmids in *B. cepacia*. Transfer of plasmids by conjugation can be demonstrated to occur from other species into *B. cepacia*, between *B. cepacia* strains and from *B. cepacia* to other species.

B. cepacia is highly variable in both genetic and phenotypic properties. There is a high degree of variability within and between clinical and environmental populations. Typing by PCR ribotyping and macrorestriction analysis showed a high level of variability in strains found in the environment throughout South Wales. This indicated 21 different strains of *B. cepacia* had been isolated, with no single strain being isolated at another sample site, or in the same sample using different isolation media. A total recovery rate of 18% from the environment was considerably higher than those of previous, more urbanised studies (Fisher *et al* 1993, Mortensen *et al* 1995), confirming *B. cepacia* is primarily an inhabitant of soils, waters and vegetation. Higher recovery rates were also found at polluted sites, suggesting *B. cepacia* fills such an ecological niche, probably as a result of its degradative properties.

Typing of isolates also indicated there is no evidence of transmission of *B. cepacia* from the local environment to CF patients in South Wales. However, there appears to be a common 'clone' within the Cardiff CF population. This 'clone' is also found at CF centres in Scotland, Ireland and France. This suggests that there is person to person transmission within the Cardiff CF population (This has previously been described by Millar-Jones *et al* [1992]), and also between patients at different CF centres. Such transmission could occur through social contact, such as CF patient meetings, or particularly with younger patients from different centres, at events such as CF summer camps.

The high levels of variation in *B. cepacia*, particularly differences in phenotypic and genetic organisation between environmental and clinical strains of *B. cepacia* add weight to suggestions for the need for greater phylogenetic division in *B. cepacia* and raises questions regarding the evolution of the forms of *B. cepacia* pathogenic to man. Differences both in clinical and environmental strains of *B. cepacia* have been used to place the species into 4 or 5 distinct groups or genomovars (Vandamme 1995, Govan *et al* 1996, Revets *et al* 1996,Vandamme *et al* 1997). The majority of environmental strains, including the type strain, fall into genomovar I, whilst the CF epidemic strain falls into genomovar III. It can be said there are fundamental differences in the properties of genomovar I and genomovar III, and it must be considered whether these are in fact the same species. The strains pathogenic to man have developed distinct mechanisms to cause infection and the pathological damage associated with the infections. This allows the *in vitro* adherence and invasion of cells, not found in environmental strains (Bevivino *et al* 1994, Burns *et al* 1996a). However such strains are unable to macerate onion tissue, something that can be achieved by many environmental strains.

It is also notable that infections in some CF patients attributed to *B. cepacia* have subsequently been found to be caused by at least 3 other *Burkholderia* species, *B. vietnamensis* and two others that cannot be differentiated by conventional means such as API 20NE (Vandamme 1995, Govan *et al* 1996). It is also significant that a multiresistant *Burkholderia* having properties of both *B. cepacia* and *B. gladioli* has been found infecting a CF patient (Simpson *et al* 1994). It was also speculated that the infective forms of *B. cepacia* may have evolved through horizontal transfer of genes

such as virulence genes from closely related species, or through the evolution of bacterial hybrids. There could be a strong argument that the ET 12 epidemic strain has features of *B. pseudomallei* , being an intracellular pathogen (Burns *et al* 1996a), and in causing a severe necrotising pneumonia (Tomashefski *et al* 1988), that is reminiscent of the necrotising condition found in meliodosis. Such evidence would indicate that '*B. cepacia*' infection may be caused by several closely related species that have different levels of infectivity and pathology and differ from 'true' *B. cepacia*, the phytopathogen of onion described by Burkholder.

The difficulty in separating these 'species' by conventional means has lead to the proposed genomovars, though as typing by a molecular basis becomes more widespread and better developed, it can be concluded that further taxonomic division of the *B. cepacia* complex within the genus *Burkholderia* is likely to occur. It is likely that *B. cepacia* should be used to describe strains that currently fall into genomovar I (phytopathogenic environmental strains like the type strain), and other genomovars renamed, hence the proposal of *B. multivorans*(Vandamme *et al* 1997). The species , several species or sub-species that infect CF patients could be renamed, possibly reflecting their properties, though as described by the authors themselves, the suggested name of '*Burkholderia cfei*' may not be entirely appropriate (Govan *et al* 1996).

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Appendix I Reagents and Media

Reagents 'Analar' or 'molecular biology grade' unless stated otherwise.

TB-T selective medium (Hagedorn *et al* 1987)

Per litre in distilled/deionised water:

20 g bacteriological agar (Oxoid ,Unipath, Basingstoke, Hants)

2 g glucose (BDH, Poole, Dorset)

1 g L-asparagine (BDH, Poole, Dorset)

1g Na HCO_3 (BDH, Poole, Dorset)

500 mg KH_2 PO_4 (BDH, Poole, Dorset)

100 mg Mg SO_4 (BDH, Poole, Dorset)

50 mg Trypan blue (BDH, Poole, Dorset)

20 mg Tetracycline (Sigma Chemical Co., Poole , Dorset)

5 mg Crystal Violet (BDH, Poole, Dorset)

Adjust to pH 5.5 with 10 % phosphoric acid (BDH, Poole, Dorset), then sterilise by autoclaving at 15 p.s.i, 121°C for 15 mins.

TE Buffer (Sambrook *et al* 1989)

Per litre in distilled/deionised water:

1.21 g Tris (hydroxymethyl)methylamine (Sigma Chemical Co., Poole, Dorset)

0.372 g EDTA(Ethylenediaminetetra-acetic acid (disodium)) (BDH, Poole, Dorset)

Adjust to pH 7.5 with NaOH, then sterilise by autoclaving at 15 p.s.i, 121 °C for 15 mins.

TBE Buffer

Per litre in distilled deionised water:

10.8 g Tris (Sigma Chemical Co., Poole, Dorset)

0.55g Boric acid (Sigma Chemical Co., Poole, Dorset)

0.74 g EDTA (BDH, Poole, Dorset)

Adjust with NaOH/NaCl to pH 8.0

Lysing solution (Kado and Liu 1981)

0.15 g Tris (Sigma Chemical Co., Poole, Dorset)

Sodium dodecyl sulphate (SDS) (Sigma Chemical Co., Poole, Dorset)

425 µl 2M NaOH (BDH, Poole, Dorset)

25 ml deionised/distilled water

Tris was dissolved in the deionised water by stirring, before the SDS was added and dissolved by stirring. The NaOH solution was added last to give a pH of 12.6. All mixing was done gently on a magnetic stirrer to prevent the SDS coming out of solution. The mixture was kept for up to 48 h at room temperature.

Set Buffer (Rodriguez and Tait 1983)

20 % w/v sucrose in distilled water (BDH, Poole, Dorset)

50 mM Tris-HCl pH 7.6 (Sigma Chemical Co., Poole, Dorset)

50 mM EDTA (BDH, Poole, Dorset)

Mix to dissolve, filter sterilise.

Lytic Mixture (Rodriguez and Tait 1983)

2 % SDS(BDH, Poole, Dorset)

0.4 M NaOH (BDH, Poole, Dorset)

Mix equal quantities before use. Store solutions at room temperature. No need to autoclave.

Ribonuclease solution (Rodriguez and Tait 1983)

10 µl of 10mM Tris at pH 7.6 (Sigma Chemical Co., Poole, Dorset)

7.5 µl of 15 mM NaCl (BDH, Poole, Dorset)

Ribonuclease A (Sigma Chemical Co., Poole, Dorset)

500µl distilled/deionised water

Mix and heat to 100 °C for 10 mins. Allow to return to room temperature slowly. Store frozen at –20°C.

Sodium acetate buffer (Rodriguez and Tait 1983)

3 M Sodium acetate (Sigma Chemical Co., Poole, Dorset)

4.92 g of sodium acetate was added to 10 ml of deionised water. The pH was adjusted to 4.8 with approximately 10 ml of glacial acetic acid (BDH, Poole, Dorset).

Alkaline dodecyl sulphate (Birnboim 1983)

1% w/v SDS in deionised water (Sigma Chemical Co., Poole, Dorset)

0.2 M NaOH (BDH, Poole, Dorset)

Mix together at appropriate concentrations. Keep at room temperature for up to a week.

Lysozyme solution (Birnboim 1983)

50 mM Glucose (BDH., Poole, Dorset)

10mM EDTA (Sigma Chemical Co., Poole, Dorset)

25 mM Tris-HCl (Sigma Chemical Co., Poole, Dorset)

Adjust pH to 8.0 with HCl/NaOH. Stores indefinitely at room temperature. Add lysozyme (Sigma Chemical Co., Poole, Dorset) at 1 mg ml^{-1} prior to use.

Acetate-MOPS solution (Birnboim 1983)

O.1 M sodium acetate (Sigma Chemical Co., Poole, Dorset)

0.05 M MOPS (morpholinopropanesulphonic acid) (Sigma Chemical Co., Poole, Dorset).

Adjust to pH 8.0 with NaOH. Store indefinitely over a drop of chloroform.

High-salt solution (Birnboim 1983)

29.4 g potassium acetate (Sigma Chemical Co., Poole, Dorset)

95 ml distilled/deionised water

5 ml of 90 % formic acid (BDH., Poole, Dorset)

Store at room temperature

Loading buffer

Bromocresol purple (0.25%) in 50% glycerol with 0.05 M tris acetate pH 7.9. Stores indefinitely at 4 °C.

Luria Bertani (LB) medium (Sambrook *et al* 1979)

Per litre:

950 ml deionised water

10g Bacteriological peptone (Oxoid, Unipath, Basingstoke, Hants)

5 g Bacteriological yeast extract (Oxoid, Unipath, Basingstoke, Hants)

10 g sodium chloride (BDH, Poole, Dorset)

Dissolve the solid and adjust to pH 7.0 with 5 M NaOH (around 0.2ml). Adjust the volume of the solution to 1 l with deionised water. Sterilise by autoclaving at 15 p.s.i, 121 °C for 15 mins.

LB-Miller medium (Qiagen Ltd. 1995)

1 l deionised water

10g Tryptone (Oxoid, Unipath, Basingstoke, Hants)

5 g Bacteriological yeast extract (Oxoid, Unipath, Basingstoke, Hants)

10 g sodium chloride (BDH, Poole, Dorset)

Prepare as for LB medium.

SE buffer (Grouthes and Tummler 1991)

4.3 g sodium chloride (BDH, Poole, Dorset)

9.3 g EDTA

Dissolve in 950 ml of deionised water. Adjust pH to 7.5 with NaOH. Make up to 1l with deionised water. Sterilise by autoclaving at 15 p.s.i, 121°C for 15 mins.

N-lauroyl sarcosinate EDTA lysing solution (Grouthes and Tummler 1991)

1 g N-lauroyl sarcosine (Sigma Chemical Co., Poole, Dorset)

18.75 g EDTA (BDH, Poole, Dorset)

Dissolve solids in 75 ml deionised water. Adjust to pH 9.5 with NaOH pellets. Adjust volume to 100 ml with deionised water. Filter sterilise , and store at 4 °C for up to 12 weeks. Prior to use add 40 µl ml^{-1} of 12.5 mg ml^{-1} proteinase K stock solution, prepared in sterile deionised water and stored at -70 °C .

Trace elements solution (Burton *et al* 1982)

Per litre in deionised water:

2.0 g $NaHCO_3 .10H_2O$

0.3 g $MnSO_4 . 4H_2O$

0.2 g $(CH_3CO_2)_2 Zn.2H_2O$

0.02 g $(NH_4)_6Mo_7O_{24} . 4H_2O$

0.5 g $CuSO_4 . 5H_2O$

0.5 g $CoCl_2 . 6H_2O$

0.2 g $Al(SO_4)_3$

0.5 g $Fe(NH_4)_2SO_4$

Appendix II Composition of reagents in commercial plasmid isolation kits

Qiagen plasmid and Qiafilter kits

Buffer P1

100 RNase A

50 mM Tris-HCl

10 mM EDTA

pH 8.0 : Store at 4 °C

Buffer P2

200 mM NaOH

1 % SDS

Store at room temperature

Buffer P3

2.55 mM Potassium acetate

pH 4.9 : Store at room temperature

Buffer QBT

750 mM NaCl

50 mM MOPS

15% ethanol

0.15 % Triton-X-100

pH 7.0 : Store at room temperature

Buffer QC

1 M NaCl

50 mM MOPS

15 % ethanol

pH 7.0 : Store at room temperature

Buffer QF

1.25 M Na Cl

50 mM MOPS

15 % ethanol

pH 8.2 : Store at room temperature

Promega Wizard plasmid miniprep kit

Cell resuspension solution

50 mM Tris-HCl

10 mM EDTA

100 µg mlRNAse A

pH 7.5

Cell lysis solution

0.2M NaOH

1 % SDS

Neutralization solution

1.32 M Potassium acetate

pH 4.8

TE Buffer

10 mM Tris -HCl

1mM EDTA

pH 7.5

Column wash solution

200 mM NaCl

20 mM Tris-HCl

5 mM EDTA

pH 7.5

Dilute with 95 % ethanol to give 55 % ethanol concentration.